

ABSTRACT

MU, XUEYAN. Anti-Ecdysteroidal Activity and Associated Toxicity of Environmental Chemicals in the Crustacean *Daphnia magna*. (Under the direction of Gerald A. LeBlanc)

The focus of this research was to characterize potential mechanisms by which environmental chemicals may disrupt ecdysteroid-regulated processes in crustaceans and the consequences of these perturbations. Several chemicals that are likely to elicit anti-ecdysteroidal activities through different mechanisms were selected and used to establish mechanisms of anti-ecdysteroidal activity.

The fungicide fenarimol was used to modulate ecdysone levels and evaluate the role of ecdysteroids in crustacean embryo development. Fenarimol lowered endogenous ecdysone levels, delayed molting, interfered with embryo development, and reduced fecundity in the crustacean *Daphnia magna*. The molting effect and embryotoxicity of fenarimol were abrogated by co-exposure to exogenous 20-hydroxyecdysone. These experiments demonstrated that ecdysteroids are critical to normal embryo development and chemicals that lower ecdysteroid levels induce developmental abnormality and adversely impact fecundity.

In chapter two, we tested the hypothesis that the known developmental toxicity of testosterone is due to anti-ecdysteroidal activity. Two ecdysteroid-regulated processes in daphnids— molting and embryo development were disrupted by testosterone. These

disruptions were mitigated by co-exposure to 20-hydroxyecdysone. However, unlike fenarimol, testosterone had no discernable effect on endogenous ecdysone levels.

Testosterone significantly antagonized the action of 20-hydroxyecdysone in an ecdysone-responsive cell line. These results demonstrated that ecdysteroid receptor antagonism could be one mechanism by which testosterone elicits embryo toxicity.

Based upon results of the first two studies, we concluded that fenarimol is an anti-ecdysteroid by reducing endogenous ecdysone levels and testosterone elicits anti-ecdysteroidal activity by functioning as ecdysteroid receptor antagonist. We next hypothesized that combined exposure to fenarimol and testosterone would result in greater than additive toxicity due to their different but interactive modes of action. Effects of fenarimol and testosterone on *ex vivo* embryo development were evaluated independently and in combination in order to detect and quantify any synergistic interactions. Synergy was observed between the two compounds. A mathematical model was developed called ‘independent joint action with fenarimol-on-testosterone synergy’ (IJA+SYN) that was highly predictive of the experimentally-determined combined effects of the two compounds.

In chapter four, we investigated the possibility that juvenoid hormones can regulate the ecdysteroid activity in daphnids in an antagonistic fashion. Juvenoids disrupted ecdysteroid-regulated embryo development in daphnids. Juvenoids also antagonized the effects of 20-hydroxyecdysone on cell proliferation and gene expression of the ecdysone receptor and its partner protein ultraspiracle in a cultured cell line. While juvenoids functioned as anti-

ecdysteroids both in vivo and in cultured cells, 20-hydroxyecdysone showed no evidence of acting as an anti-juvenoid. We concluded that the ability of juvenoids to block ecdysteroid-mediated induction of the ecdysone receptor and ultraspiracle could partially explain the anti-ecdysteroidal activities of these compounds. However, demonstrated synergistic interactions between a juvenoid and fenarimol could not be explained based upon this mode of activity. We concluded additionally, unidirectional properties of juvenoids contribute to their anti-ecdysteroidal activity.

Having identified and characterized several mechanisms by which chemicals can elicit anti-ecdysteroidal activity, we lastly investigated the mechanism by which the environmental contaminant bisphenol A elicits toxicity. We discovered that bisphenol A elicit juvenoid-like activity and likely interfere with ecdysteroid regulated processes via this activity. A definitive assessment revealed that bisphenol A is chronically toxic to daphnids, probably through its anti-ecdysteroidal/juvenoid activity. However, effects are elicited at levels that are not likely to pose environmental concern.

Overall, results of this study provide conclusive evidence that diverse environmental chemicals can elicit anti-ecdysteroidal activity in crustaceans through several mechanisms and result in developmental abnormalities and reduced fecundity. Such effects of environmental contaminants should be considered when evaluating causes of population decline among economically-important crustacean populations.

**ANTI-ECDYSTEROIDAL ACTIVITY AND
ASSOCIATED TOXICITY OF ENVIRONMENTAL CHEMICALS IN
THE CRUSTACEAN *DAPHNIA MAGNA***

by
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*This work is dedicated to my wife, my parents, and my sisters
for their endless love and support.*

BIOGRAPHY

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The author married Xiao Zhuang in 1998 in Qingdao, China. When writing this dissertation, he and his wife are expecting their first child.

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TABLE OF CONTENTS

	Page
LIST OF TABLES	viii
LIST OF FIGURES.....	ix
INTRODUCTION.....	1
References.....	11
CHAPTER 1 - ENVIRONMENTAL ANTIECDYSTEROIDS ALTER	
EMBRYO DEVELOPMENT IN CRUSTACEAN <i>Daphnia magna</i>	20
Abstract.....	21
Introduction.....	22
Materials and Methods	23
Results and Discussion.....	26
Acknowledgement.....	29
Literature cited.....	30
Figure Legends.....	34
CHAPTER 2 - DEVELOPMENTAL TOXICITY OF TESTOSTERONE IN	
THE CRUSTACEAN <i>Daphnia magna</i> INVOLVES ANTI-ECDYSTEROIDAL	
ACTIVITY	41
Abstract.....	42
Introduction.....	43
Materials and Methods	45

Results.....	48
Discussion.....	52
Acknowledgement.....	55
References.....	56
Figure Legends.....	63
CHAPTER 3 - SYNERGISTIC INTERACTION OF ENDOCRINE DISRUPTING	
CHEMICALS: MODEL DEVELOPMENT USING AN ECDYSONE RECEPTOR	
ANTAGONIST AND A HORMONE SYNTHESIS INHIBITOR	
Abstract.....	71
Introduction.....	72
Materials and Methods	74
Results.....	77
Discussion.....	82
Acknowledgement.....	86
References.....	89
Figure Legends.....	90
CHAPTER 4 - ANTI-ECDYSTEROIDAL ACTIVITY OF JUVENOID	
HORMONES IN A CRUSTACEAN.....	
Abstract.....	106
Introduction.....	107
	108

Materials and Methods	111
Results.....	116
Discussion.....	119
References.....	123
Figure Legends.....	129
CHAPTER 5 - ANTIECDYSTEROIDAL ACTIVITY OF BISPHENOL A TO A	
CRUSTACEAN <i>Daphnia magna</i>	138
Abstract.....	139
Introduction.....	140
Materials and Methods	142
Results.....	148
Discussion.....	151
Acknowledgement.....	153
References.....	154
Figure Legends.....	161
SUMMARY AND CONCLUSIONS.....	171
References.....	177

LIST OF TABLES

	Page
Chapter 1	
Table 1. Ecdysone levels in daphnids that were exposed to Fenarimol during the first intermolt period or during ex vivo embryonic development.....	33
Chapter 2	
Table 1. Performance of daphnids exposed to 8.0 μ M of vertebrate-type steroid hormones for 3 weeks.....	61
Table 2. Ecdysone levels in daphnids that were exposed to 25 μ M testosterone during the first intermolt period.....	62
Chapter 3	
Table 1. Influence of one antiecdysteroid on the concentration-response curve of the other.....	97
Chapter 5	
Table 1. Susceptibility of variously aged embryos to the developmental toxicity of bisphenol A.....	160

LIST OF FIGURES

	Page
 Chapter 1	
Figure 1. Influence of fenarimol and 20-hydroxyecdysone on molting as measured by the duration of the first and second intermolt periods.	36
Figure 2. Influence of 20-hydroxyecdysone on the fenarimol-induced increase in intermolt period duration.....	37
Figure 3. Developmental abnormalities among embryonic daphnids resulting from fenarimol exposure.	38
Figure 4. Ablation of fenarimol-induced developmental abnormalities by 20-hydroxyecdysone during ex vivo embryo exposure	39
Figure 5. Total offspring produced by maternal daphnids during exposure to concentrations of fenarimol.....	40
 Chapter 2	
Figure 1. Influence of testosterone on molting as measured by the duration of the first intermolt period.....	65
Figure 2. Influence of 20-hydroxyecdysone on the testosterone-induced increase in the duration of the first intermolt period.....	66
Figure 3. Chronic toxicity resulting from maternal testosterone exposure. a: Incidence of developmental abnormalities resulting from maternal exposure to concentrations of testosterone. b: Total offspring produced by maternal daphnids during exposure to concentrations of testosterone.....	67

Figure 4. Mitigation of testosterone-induced developmental abnormalities by 20-hydroxyecdysone during in vitro embryo exposure.....	68
Figure 5. Concentration-response effects of 20-hydroxyecdysone in Kc cells on (a) cell proliferation, and (b) cell morphological response.....	69
Figure 6. Antagonistic activity of testosterone on the action of 20-hydroxyecdysone in Kc cells.....	70

Chapter 3

Figure 1. Ecdysone levels in daphnid embryos during development.....	100
Figure 2. Ecdysone levels in juvenile daphnids at various times between the first and second molt.....	101
Figure 3. Differential effects of fenarimol and testosterone on embryo development.....	102
Figure 4. Developmental abnormalities associated with exposure to fenarimol or testosterone.....	103
Figure 5. Incidence of developmental abnormalities resulting from direct embryonic exposure to concentrations of fenarimol and testosterone.....	104
Figure 6. Comparison of observed embryo toxicity of 30 binary combinations of fenarimol and testosterone with the predicted toxicity based upon the independent joint action (IJA) model (A) and the IJA + synergy (IJA+SYN) model (B).....	105

Chapter 4

Figure 1. Incidence of developmental abnormalities resulting from in vitro embryo exposure to concentrations of juvenoids.....	131
Figure 2. Developmental abnormality associated with exposure to juvenoids.....	132
Figure 3. Antagonistic activity of pyriproxyfen on the anti-proliferative action of 20-hydroxyecdysone on Kc cells.....	133
Figure 4. Influence of pyriproxyfen on the 20-hydroxyecdysone-induced gene expression of (A) ecdysone receptor and (B) ultraspiracle.....	134
Figure 5. Effect of 20-hydroxyecdysone on juvenoids-driven male-sex determination during oocyte exposure.....	135
Figure 6. The EC50 isobole for pyriproxyfen and fenarimol mixtures resulting from in vitro embryo exposure.....	136
Figure 7. Some proposed mechanisms by which juvenoids could stimulate receptor dimerization between USP and HR38 or USP and MfR resulting in receptor partner (USP) deprivation to the ecdysteroid receptor (EcR) and loss of EcR signaling activity.....	137

Chapter 5

Figure 1. Intermolt duration between the first and second molt of neonatal daphnids exposed to concentrations of bisphenol A.....	164
Figure 2. Percentage neonates with developmental abnormalities during	

exposure to concentrations of bisphenol A.....	165
Figure 3. Influence of 20-hydroxyecdysone exposure on delayed molting caused by bisphenol A.....	166
Figure 4. Influence of 20-hydroxyecdysone exposure on developmental abnormalities caused by bisphenol A.....	167
Figure 5. Incidence of male-sex determination among offspring produced by maternal daphnids exposed to methyl farnesoate, bisphenol A, or combinations thereof.....	168
Figure 6. Incidence of male-sex determination among offspring produced by maternal daphnids exposed to methyl farnesoate, methoprene, or combinations thereof.....	169
Figure 7. Total offspring produced among daphnids continuously exposed to concentrations of bisphenol A for 21 days.....	170

INTRODUCTION

Many environmental chemicals have been shown to elicit toxicity by disrupting the normal function of the endocrine system in both wildlife and humans (Guillett and Crain, 1999). These chemicals, termed endocrine disrupting compounds (EDCs), include a wide variety of pesticides, pharmaceuticals and industrial chemicals. A great deal of research has focused on the impact of EDCs on vertebrates such as fish (Folmar et al., 1996), reptiles (Crain and Guillette, 1998), and amphibians (Hayes et al., 2002). Little is known about the effects of EDCs on invertebrates. Invertebrates comprise approximately 95% of all the known animal species (Barnes, 1968), they are ecologically indispensable in the trophic transfer of nutrients and carbon. Many invertebrate species also can serve as biological sentinels of environmental insult. Identification and characterization of the effects of EDCs on invertebrate species may prove useful in recognizing and lessening such effect before effects are elicited in vertebrate populations, including humans.

Crustacean populations are of vital economic and ecological significance worldwide. The abundance of some economically important crustacean populations (i.e. blue crab, brown shrimp) reportedly declined or remained suppressed through the 1990s in relation to previous years. In contrast, many finfish populations in the same locations had remained stable over the same time period (Land et al., 1995, 1996 a, b). Coastal pollution is commonly cited as a possible contributing cause for declining crustacean populations (Hoss and Hettler, 1979, Reyes et al., 1999). Studies in wildlife populations have demonstrated that environmental

contaminants often impact population abundance by interfering with some aspect of endocrine signaling resulting in aberrations in development, reproduction, or other endocrine-regulated processes (Guillette and Crain, 1999). Crustaceans and other arthropods utilize ecdysteroids, an invertebrate unique hormone, as major endocrine signaling molecules (LeBlanc et al., 1999) and interference with ecdysteroid production or function could provide a means by which environmental chemicals impact crustacean species while not affecting vertebrates.

The ecdysteroids constitute a family of polyhydroxylated steroid hormones that are essential in the regulation of molting in arthropods (LeBlanc et al., 1999). Biosynthesis of ecdysteroids occurs primarily in the prothoracic glands of the insects and the Y-organ of crustaceans. The molting process is triggered by an increase in the titer of 20-hydroxyecdysone (20E), the physiologically active form of arthropod molting hormone, and is completed following the decline of 20E titer and the release of a series of neuropeptides that complete ecdysis (Chang, 1989, Nijhout, 1998). Ecdysteroids also are involved in reproduction and embryonic development in insects. The follicle cells of the ovary in insects synthesize various ecdysteroids, most of which are incorporated into the eggs as conjugates to be used for embryonic molts before the prothoracic glands of the prelate becomes functional (Sbrenna, 1991). Ecdysteroids also stimulate vitellogenin synthesis and ovarian maturation in some insects (Raabe, 1986). Considerably less is known of the role of ecdysteroids in crustacean reproduction. However, mature females have been shown to have higher circulating ecdysteroid levels as compared to males suggesting a role for these

hormones in female reproductive processes (Cuzin-Roudy et al., 1989). Ecdysteroids conjugates are transferred from the ovary to the eggs of some crustacean where they presumably regulate aspects of early embryonic development (Chaix et al., 1982). Ecdysteroid levels tend to rise in the developing embryo which suggest that the embryo is capable of ecdysteroid synthesis and ecdysteroids are required for continued normal embryonic development (Wilder and Aida, 1995).

Many environmental chemicals can elicit adverse effects on molting, reproduction and embryo development in crustaceans. For examples, molting is inhibited by heavy metals (Weis et al., 1992), polychlorinated biphenyls (PCBs) (Fingerman and Fingerman, 1977), and benzene (Cantelmo et al., 1981), and vertebrate estrogens (Baldwin et al., 1995, Zou and Fingerman, 1997). Toxicant effects on the development or reproduction have also been reported. Exposure of the amphipod *Melita nitida* to sediments contaminated with waste crankcase oil caused abnormalities in oostegite setae (Borst et al., 1987). Certain environmental chemicals (4-nonylphenol, propiconazole, piperonyl butoxide) have been revealed to interfere with the development of embryonic daphnids (*Daphnia magna*) resulting in the arrest of certain developmental processes (Kast-Hutcheson et al., 2001, LeBlanc et al., 2000). But most these studies have been concerned primarily with morphological and temporal effects of pollutant exposure. Little research has been conducted to exam the cellular or molecular effect of these chemicals on the crustacean hormonal system. Should ecdysteroids regulate molting and embryo development in crustaceans, then

environmental chemicals disrupting ecdysteroid activities could elicit adverse effect on molting and embryo development.

Little research has been done to exam the ecdysteroid disrupting activities of environmental chemicals in crustacean. The disruption in ecdysteroid regulated processes in insects is better understood because of the need to control insect pests (LeBlanc et al., 1999). Effects of the insect ecdysteroids can be disrupted by inhibition of synthesis and/release from the prothoracic glands, interference with the metabolism of 20E, or by competition at its receptor level. Because of the remarkable similarities between the endocrinology of insects and crustaceans (Lafont, 2000), it is likely that any chemical that disrupt the normal ecdysteroid control in insects will also be active in crustacean.

Azadirachtin, an insect antifeedant, has been shown to dramatically reduce the titers of 20E in several insects (Mordue et al., 1993). A variety of morphological effects of azadirachtin have been observed including growth inhibition, malformation of developmental stages, and lethal nymphal or larval molts. The ecdysone biosynthesis pathway comprises several steps catalyzed by cytochrome P-450 monooxygenases. Many environmental chemicals (e.g. Fenarimol, propiconazole etc.) are known to inhibit cytochrome P450 enzymes and may accordingly interfere with ecdysone synthesis (Grieneisen et al., 1993, Warren et al., 1995, Williams et al., 2000) and elicit anti-ecdysteroidal activities in crustaceans.

The interaction of a compound at the ecdysone receptor can be either agonistic or antagonistic. If agonistic, the compound will mimic the action of ecdysteroid, if antagonistic, it will block the ecdysteroid activity by preventing binding of endogenous ecdysteroid to the receptor. Environmental chemicals have the potential to function as ecdysone agonist by binding to and activating the ecdysone receptor (EcR). Many plant compounds are known to function as ecdysone receptor agonists (Dinan et al., 2001a) and some insecticides function by acting as ecdysteroids (Sundaram et al., 1998). Ecdysteroid agonists have been shown to accelerate molting, cause incomplete ecdysis, and cause death during molting (Clare et al., 1992, Baldwin et al., 2001). Using an ecdysteroid-responsive *Drosophila* B_{II} cell line combined with an EcR binding assay, Dinan et al. (2001b) screened about 80 potential environmental contaminants and found that the vast majority of these xenobiotics and vertebrate steroids showed neither agonist nor antagonist activity. The few that had affinity to the EcR were found to be EcR antagonist. Among those EcR antagonists, diethylphthalate and 4-nonylphenol have previously been shown to delay molting and disrupt embryo development in crustaceans (Zou and Fingerman, 1997, Kast-Hutcheson *et al.*, 2001).

The effect of ecdysteroids can also be modulated by juvenile hormone (JH) in insects. For example, in the presence of JH, ecdysteroid pulses cause larval-larval molts; but ecdysteroid triggers larval-pupal, then pupal-adult metamorphosis in the absence of JH (Riddiford, 1994). Juvenile hormone analogs (JHAs) represent a class of insecticides that designed specifically disrupt insect development and/or reproduction without impinging on physiological processes in other animals where JH is not present and has no known function

(Dhadialla et al., 1998). In insects, exposure to JHAs during early embryonic development or final larval stage disrupts development and results in deformed adults or compromised metamorphosis (Riddiford, 1994, Staal, 1975). Cherbas et al. (1989) reported that JHA methoprene antagonized the effects of 20E in the ecdysone-responsive *Drosophila* Kc cells. JHAs also have been reported to reduce fecundity (Mckenney and Celestial, 1996; Olmstead and LeBlanc, 2001) and interfere with larval development (Cripe et al., 2003) in crustaceans. JHAs might elicit these adverse effects in crustaceans by interfering with the regulatory activity of ecdysteroids.

Molecular studies in insects provide addition support for the hypothesis of the anti-ecdysteroid activity of JHAs. Ecdysteroid action at the molecular level has been well studied in insects, especially *Drosophila melanogaste*. 20-Hydroxyecdysone (20E), the biologically active ecdysteroid, binds to the ecdysone receptor (EcR) when it forms a heterodimer with ultraspiracle (USP). The hormone-receptor complex then binds to the ecdysone response element to active or inactivate gene transcription (Yao et al., 1993, Cherbas and Cherbas, 1996). JH has been found to bind to USP with low affinity and to promote its homodimerization in a yeast system (Jones and Sharp, 1997). Other nuclear receptors (e.g. DHR 38, AaSvp) can heterodimerize with USP, disrupt the EcR and USP dimerization, and interfere with the ecdysteroid signal transduction pathway (Sutherland et al., 1995, Zhu et al, 2003). If the expression of these receptors is under the control of juvenoid hormone, then this disruption of EcR-USP heterodimerization could provide a mechanism for anti-ecdysteroidal activity of juvenoids.

Organisms are rarely exposed to individual chemicals in the environment. Endocrine toxicants may harbor an increased likelihood for non-additive interactions owing to their potential to elicit effects at different targets along a common signaling pathway. Most often, the consequences of such interactions will conform to a model for independent joint action where effects of different chemicals are elicited at independent targets though a common outcome occurs (Bliss, 1939, Broderius et al., 1995). However, scenarios could arise whereby one endocrine toxicant modifies the potency of another resulting in either an antagonistic or synergistic effect. Indeed, synergistic effects of endocrine toxicants have been reported, though the mechanisms responsible for these non-additive interactions typically have remained elusive (Kortenkamp and Alterburger, 1998, Rajapakse et al., 2002). A crustacean model and anti-ecdysteroids with different modes of action could be used to address some fundamental issues in the toxicology of chemical mixtures. Results also may be directly relevant to the reported decline of some crustacean populations. Several antiecdysteroids, including bisphenol A, lindane, dieldrin, and 4-nonylphenol (Dinan et al., 2001b), were among the chemicals detected with 3 to 51% frequency among 139 streams sampled during 1999-2000 and analyzed for 95 contaminants (Kolpin et al., 2002). It is possible that some crustacean populations have experienced reduced fecundity from exposure to antiecdysteroids at concentrations of the individual chemicals previously deemed safe.

In this research, the hypothesis was tested that environmental antiecdysteroids can disrupt normal embryo development and compromise the production of viable offspring in

crustacean *Daphnia magna*. Several potential mechanisms by which chemicals may elicit antiecdysteroidal activity were considered in this research.

In chapter one, agricultural fungicide fenarimol, a known cytochrome P450 enzyme inhibitor, was used as a probe to evaluate the role of ecdysteroids in crustacean embryo development. We hypothesized that this compound would inhibit ecdysteroid synthesis, disrupt molting, and interfere with embryo development in daphnids. To test this hypothesis, the effects of fenarimol on molting and endogenous ecdysteroid levels were first evaluated. Then co-exposure experiments involving both fenarimol and 20-hydroxyecdysone were used to confirm the anti-ecdysteroid activity of fenarimol. Next, we evaluated the implications of the anti-ecdysteroid activity of fenarimol to embryo development in both maternal and isolated embryo exposure experiments. The effect of fenarimol on the fecundity of the parental organisms was also determined.

In chapter two, we tested the hypothesis that the well-characterized adverse effects of testosterone on the developing daphnid embryo are due to anti-ecdysteroidal properties of this compound. This hypothesis was tested first by evaluating the relationships between testosterone exposure levels and two ecdysteroid-regulated processes in daphnids— molting and embryo development. Secondly, the involvement of anti-ecdysteroidal activity of testosterone on molting and embryo development was evaluated by determining the ability of exogenously administered 20-hydroxyecdysone to protect against the toxicity of testosterone.

Lastly, the mechanism by which testosterone elicits anti-ecdysteroidal activity was determined.

Based upon results of the first two studies, we concluded that fenarimol is an ecdysteroid synthesis inhibitor and testosterone elicits anti-ecdysteroidal activity by functioning as ecdysteroid receptor antagonist. In chapter three, we tested the hypothesis that a hormone synthesis inhibitor and a hormone receptor antagonist would *synergize* to yield greater toxicity than that predicted from a model of independent joint action. Effects of fenarimol, an ecdysteroid synthesis inhibitor and testosterone, an ecdysteroid receptor antagonist on ex vivo embryo development were evaluated independently and in combination in order to detect and quantify any synergistic interactions. Then, mathematical models were developed based on the established modes of antiecdysteroidal activity and synergy of these two compounds and model predictions were tested experimentally.

In chapter four, we hypothesized that juvenoids can disrupt embryo development, and elicit anti-ecdysteroidal activity in daphnids. This hypothesis was tested first by determining if juvenoids, in general, can disrupt ecdysteroid-regulated embryo development in daphnids. Secondly, the anti-ecdysteroidal activity of juvenoids and associated mechanism were evaluated using both in vivo approaches and an ecdysteroid-responsive cell line.

In chapter five, we investigated the anti-ecdysteroidal activity of the ubiquitous environmental contaminant bisphenol A. The ability of bisphenol A to interfere with two

ecdysteroid-dependent physiological processes – molting and embryo development - was first evaluated. Based upon the results from chapter four, Juvenoids are known to function as anti-ecdysteroid. Therefore, the ability of bisphenol A to elicit juvenoid activity was evaluated secondly. Lastly, the definitive assessment of the effects of bisphenol A on the reproductive capacity of daphnids was conducted to determine if this contaminant is likely to pose an threat at levels expected to occur in the environment.

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**ENVIRONMENTAL ANTIECDYSTEROIDS ALTER EMBRYO DEVELOPMENT
IN CRUSTACEAN *Daphnia magna***

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ABSTRACT

The role of ecdysteroids in crustacean embryo development and the susceptibility of the developing embryo to the antiecdysteroidal properties of an environmental chemical were evaluated. The agricultural fungicide fenarimol was shown to exhibit antiecdysteroidal activity to the crustacean *Daphnia magna* by lowering endogenous ecdysone levels and delaying molting in a concentration-dependent fashion that was abrogated by co-exposure to exogenous 20-hydroxyecdysone. Exposure of either gravid maternal organisms or isolated embryos to fenarimol resulted in embryo abnormalities ranging from early partial developmental arrest to incomplete development of antennae and shell spines.

Developmental abnormalities were associated with suppressed ecdysone levels in the embryos and the abnormalities could be prevented by co-exposure to 20-hydroxyecdysone. Developmental abnormalities caused by the antiecdysteroid were associated with reduced fecundity of the parental organisms. These results demonstrate that ecdysteroids are critical to normal crustacean embryo development and environmental antiecdysteroids can disrupt normal embryo development and compromise the production of viable offspring.

Antiecdysteroidal activity may provide a means by which environmental chemicals impact crustacean species while not affecting vertebrates.

INTRODUCTION

Crustacean populations are of vital economic and ecological significance worldwide. The abundance of some economically important crustacean populations (i.e. blue crab, brown shrimp) reportedly declined or remained suppressed through the 1990s in relation to previous years. In contrast, many finfish populations in the same locations had remained stable over the same time period (Land et al., '95, '96 a, b). Coastal pollution is commonly cited as a possible contributing cause for declining crustacean populations (Hoss and Hettler, '79, Reyes et al., '99).

Mortality of blue crabs resulting from organochlorine insecticide exposure had been noted following decades of use of these chemicals (Van Engel, '82). However, the use of organochlorine insecticides has been significantly curtailed in the United States over the past thirty years and they are typically no longer abundant contaminants along the coastal United States. In contrast, herbicides and fungicides are among the dominant contemporary agricultural organic contaminants and relatively little is known of the impact of these chemicals on crustaceans.

Studies in wildlife populations have demonstrated that environmental contaminants often impact population abundance by interfering with some aspect of endocrine signaling resulting in aberrations in development, reproduction, or other endocrine-regulated processes (Guillette Jr. and Crain, '99). Crustaceans and other arthropods utilize ecdysteroids as major

endocrine signaling molecules (LeBlanc et al., '99) and interference with ecdysteroid production or function could provide a means by which environmental chemicals impact crustacean species while not affecting vertebrates. While ecdysteroids are known to regulate the molting processes in crustaceans (LeBlanc et al., '99), little is known regarding other functions of these hormones in crustaceans. Fenarimol (α -(2-chlorophenyl)- β -(4-chlorophenyl)-5-pyrimidinemethanol) is an agricultural fungicide that is known to inhibit cytochrome P450 enzymes and may accordingly interfere with ecdysone synthesis (Grieneisen et al., '93, Warren et al., '95, Williams et al., '00). We hypothesized that this compound would inhibit ecdysteroid synthesis in *Daphnia magna*, a crustacean species that is commonly used in toxicity evaluations, and would serve as a probe to evaluate the role of ecdysteroids in crustacean embryo development.

METHODS AND MATERIALS

Effects of fenarimol or/and 20-hydroxyecdysone on molting as measured by the duration of the first and second intermolt periods. Individual neonatal (<1 hour old) daphnids were exposed to concentrations of fenarimol (Chem Service, West Chester, PA) or/and 20-hydroxyecdysone (Sigma, St. Louis, MO) and the duration of the first and second intermolt periods was measured to the nearest hour. Daphnids were cultured and assayed as described previously (LeBlanc et al., '00). Fenarimol and 20-hydroxyecdysone solutions were prepared in absolute ethanol (AAPER, Shelbyville, KY) and all exposure solutions, including controls, contained 0.01% ethanol. Daphnids were individually exposed to 40 mL

of test solution in 50 mL beakers and were transferred to newly prepared exposure solutions every 24 hours.

Embryotoxicity of fenarimol: maternal exposure The incidence of developmental abnormalities in embryos resulting from maternal exposure to concentrations of fenarimol was assessed. Individual female daphnids (<1 hour old) were exposed to concentrations of fenarimol as described above. These exposure conditions favor parthenogenetic reproduction. Daphnids were reared to maturity (~6 days), then offspring production was assessed daily and individual offspring were evaluated microscopically to establish survival and to identify any developmental abnormalities. Experiments were terminated after 21 days of exposure. Developmental abnormalities were documented using a digital camera (Pixera Corporation, Los Garos, CA) affixed to the microscope.

Embryotoxicity of fenarimol: direct embryo exposure Embryos that were in early stages of development (developmental stage 1 as defined previously (Kast-Hutcheson et al., '01)), were removed from untreated daphnids as described previously (LeBlanc et al., '00). Embryos were individually and randomly assigned to wells of 96-well microtiter plates along with 200 µL of medium containing the desired concentration of fenarimol. All embryos, including controls, were exposed to the same concentration of ethanol vehicle (0.01%). Embryos were incubated at 20⁰C under a 16 hr photoperiod and were examined microscopically every 24 hrs. Embryos were scored for stage of development and any abnormalities were noted. The percentage of embryos that exhibited developmental

abnormalities was determined at the time at which development of the control daphnids was complete.

20-Hydroxyecdysone rescue: The ability of exogenous 2-hydroxyecdysone to prevent fenarimol-induced embryo toxicity was evaluated. Isolated embryos were individually exposed to 4.0 μ M fenarimol as described above along with increasing concentrations of 20-hydroxyecdysone. Embryo toxicity was evaluated as described above.

Ecdysone radioimmunoassay. Ecdysone levels in daphnids were measured by radioimmunoassay as described previously (Chang and O'Connor, '79, Warren and Gilbert, '88). The ecdysone antiserum was produced by Dr. W.E. Bollenbacher (University of North Carolina, Chapel Hill, NC) and distributed by Dr. E.S. Chang (University of California, Bodega Marine Laboratory, Bodega Bay, CA). The radioimmunoassay was validated for use with the daphnid preparations as we have described for other antibodies and matrices (Parks et al., '99).

Statistics: Statistical significance was evaluated by Analysis of Variance and Dunnett's *t*-test when comparing multiple treatments to the control and by Student's *t*-test when comparing a single treatment to the control using JMP software (SAS Institute, Cary, NC). Concentration–response curves were generated by linear regression or sigmoidal fit using Origin software (Microcal Software Inc., Northampton, MA).

RESULTS AND DISCUSSION

The ability of fenarimol to act as an antiecdysteroid was first evaluated by assessing its impact on molting. Exposure of neonatal daphnids to fenarimol increased the time required for the organisms to undergo both the first and second molt in a fenarimol concentration-dependent manner (Fig. 1 a & b). These results were consistent with fenarimol functioning as an antiecdysteroid either by lower endogenous ecdysteroid levels or inhibiting the activity of endogenous ecdysteroid. Radioimmunoassay of fenarimol-exposed neonatal daphnids revealed that ecdysone levels had been significantly reduced in individual organisms by approximately 26% (Table 1a). This reduction also was evident when ecdysone levels were normalized to the dry weight of the daphnids (29% reduction). The ability of fenarimol to delay molting by acting as an antiecdysteroid was confirmed during co-exposure experiments involving both fenarimol and 20-hydroxyecdysone, a ligand to the ecdysone receptor. Exposure of daphnids to 20-hydroxyecdysone, alone, had no significant effect on the time required for the organisms to molt at the concentrations evaluated (Fig. 1 c & d). However, exposure to increasing concentrations of 20-hydroxyecdysone progressively mitigated the delay in molting caused by fenarimol (Fig. 2). Interestingly, 20-hydroxyecdysone was able to completely rescue the daphnids from the effects of fenarimol on molting during the first intermolt period, but had little effect during the second intermolt period. This difference in effectiveness may be due to induction of ecdysteroid biotransformation enzymes or other inactivation/elimination processes in response to the increase in ecdysteroid levels during the first intermolt period.

Having established that fenarimol does indeed elicit antiecdysteroid activity in a crustacean, we next evaluated the implications of this activity to embryo development. Exposure of maternal daphnids to fenarimol caused a concentration-dependent increase in developmental abnormalities in offspring (Fig. 3a). Developmental effects ranged from minor abnormalities of the carapace and antennae to severely developmentally-arrested individuals (Fig. 3b-f).

The mechanism by which fenarimol interfered with normal development was investigated using daphnid embryos cultured *in vitro* (LeBlanc et al., '00). Direct incubation of daphnid embryos with 4.0 μ M fenarimol resulted developmental abnormalities that were consistent with those observed during maternal exposure (Fig. 3b-f). In addition, fenarimol exposure during embryo development resulted in a significant decrease in the ecdysone levels in the neonatal organisms (Table 1b). This decrease was evident when ecdysone levels were quantified on either a per neonate basis (58% decrease) or a dry weight basis (48% decrease). The developmental abnormalities associated with fenarimol exposure could be directly ascribed to the decrease in ecdysone levels since these developmental abnormalities were abrogated by co-exposure to 20-hydroxyecdysone (Fig. 4). Exposure of embryos to 20-hydroxyecdysone alone had no discernable effect on embryo development (Fig. 4).

The implications of fenarimol-induced developmental toxicity on fecundity was evaluated by exposing individual parthenogenetically-reproducing daphnids to

concentrations of fenarimol and evaluating the production of viable offspring. Individual control daphnids produced approximately 100 offspring over the course of the experiment. Commensurate with the occurrence of developmental abnormalities (i.e., Fig. 3a), progressively fewer viable offspring were produced with increasing fenarimol concentration (Fig. 5). Thus, the developmental abnormalities that occurred as a consequence of the antiecdysteroid activity of fenarimol, could significantly and adversely affect population abundance.

These results clearly demonstrate that ecdysteroids are critical to normal crustacean embryo development and environmental antiecdysteroids can disrupt normal embryo development and compromise the production of viable offspring. Whether environmental chemicals with antiecdysteroidal activity actually have impacted crustacean populations remains to be established. This possibility rests in the degree to which this property is shared among environmental chemicals and the concentrations at which these chemical exist in the environment.

Other fungicides such as the imidazole fungicides also are known to inhibit cytochrome P450 activity and accordingly may interfere with ecdysteroid biosynthesis. We recently have demonstrated the one such fungicide, propiconazole, alters daphnid embryo development in a manner consistent with it functioning as an antiecdysteroid (Kast-Hutcheson et al., '01). We also have shown that 4-nonylphenol, a relatively common environmental contaminant (Talmage, '94, Weeks et al., '98), elicits developmental toxicity to daphnids consistent with that of an antiecdysteroid (LeBlanc et al., '00). The apparent

ubiquity of antiecdysteroidal activity of environmental chemicals necessitates investigation into whether environmental antiecdysteroids are present either alone or in combination at concentrations sufficient to impact coastal crustacean populations.

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Table 1 Ecdysone levels in daphnids that were exposed to fenarimol during the first intermolt period (a) or during ex vivo embryonic development (b). A asterisk denotes a significant difference from the respective control (0 μ M fenarimol) at $p \leq 0.05$ (Student's t test).

Experiment	Fenarimol (μ M)	Ecdysone (pg/neonate)
a: neonatal exposure	0	28.7 ± 7.0^a
	1.5	$21.1 \pm 5.5^*$
b: embryonic exposure	0	48.8 ± 13.1
	4.0	$20.6 \pm 7.4^*$

^amean $\pm$ standard deviation; n = 5-13 groups with each group containing 25-75 individual organisms.

FIGURE LEGENDS

Figure 1. Influence of fenarimol (a, b) and 20-hydroxyecdysone (c, d) on molting as measured by the duration of the first (a, c) and second (b, d) intermolt periods. The dotted lines represent the mean duration of the intermolt period of 10 daphnids exposed to neither fenarimol nor 20-hydroxyecdysone. The duration of the intermolt periods significantly increased with increasing fenarimol concentration (a: first intermolt period, $r=0.81$, $p=0.0004$; b: second intermolt period, $r=0.93$, $P<0.0001$). Increasing 20-hydroxyecdysone concentrations had no significant effect on the duration of the intermolt periods (c: first intermolt period, $r=-0.37$, $p=0.10$; d: second intermolt period, $r=-0.29$, $p=0.21$).

Figure 2. Influence of 20-hydroxyecdysone (20E) on the fenarimol-induced increase in intermolt period duration. Neonatal daphnids (<1 hour old) were individually exposed to 1.5 μM fenarimol alone or in combination with concentrations of 20E. Treatments are: control () , 1.5 μM fenarimol alone() , 1.5 μM fenarimol + 0.05 μM 20E () , 1.5 μM fenarimol + 0.16 μM 20E () , 1.5 μM fenarimol + 0.50 μM 20E (). The duration of the first and second intermolt period was measured as described in the methods. Data are presented as mean and standard deviation ($n=10$). An asterisk denotes a significant difference from the respective control at $p\leq 0.05$ (ANOVA, Dunnett's t test).

Figure 3. Developmental abnormalities among embryonic daphnids resulting from fenarimol exposure. a: Incidence of developmental abnormalities resulting from maternal exposure to

concentrations of fenarimol. The open circle represents the incidence of developmental abnormalities among offspring produced by ten control daphnids. b: normal (control) neonatal daphnid. Arrows identify the areas (antennae and shell spine) that most commonly exhibiting abnormalities. c-f: developmental abnormalities resulting from fenarimol exposure. Daphnids depicted in micrographs c-f are the same age as the control neonate depicted in 'b'.

Figure 4. Ablation of fenarimol-induced developmental abnormalities by 20-hydroecdysone (20E) during ex vivo embryo exposure. Data are presented as the mean $\pm$ standard deviation (n=5-6 groups per treatment. Each group consisted of 40-75 embryos). An asterisk denotes a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's t test).

Figure 5. Total offspring produced by maternal daphnids during exposure to concentrations of fenarimol. Each data point represents the total number of offspring produced by a single parthenogenetically reproductive female over a 21 day exposure period. Performance of control daphnids (mean $\pm$ standard deviation, n=10) is depicted as the open circle with error bars.

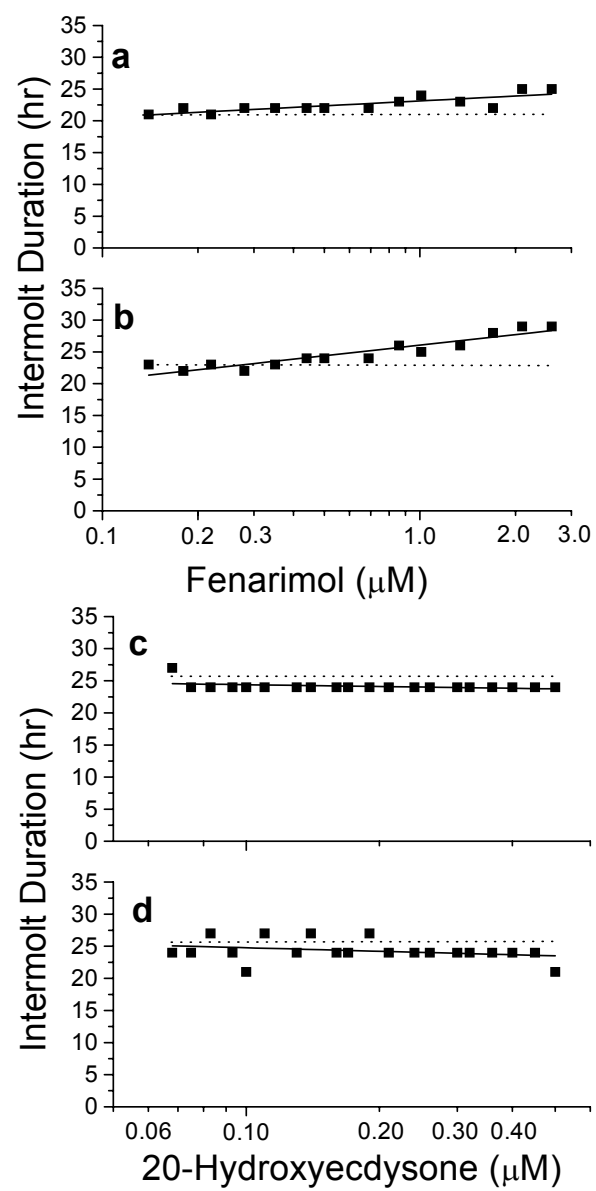


Figure 1

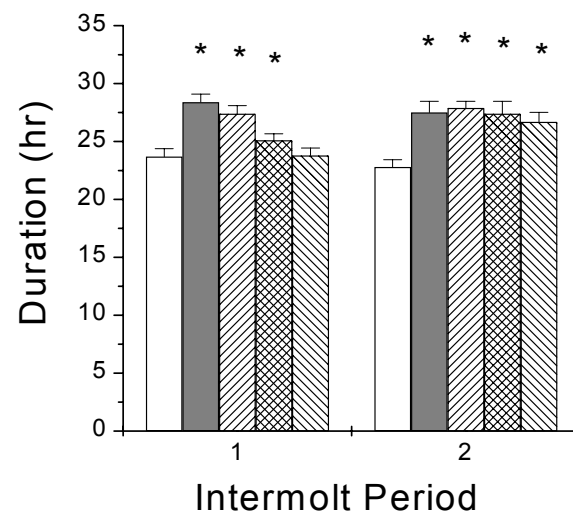


Figure 2

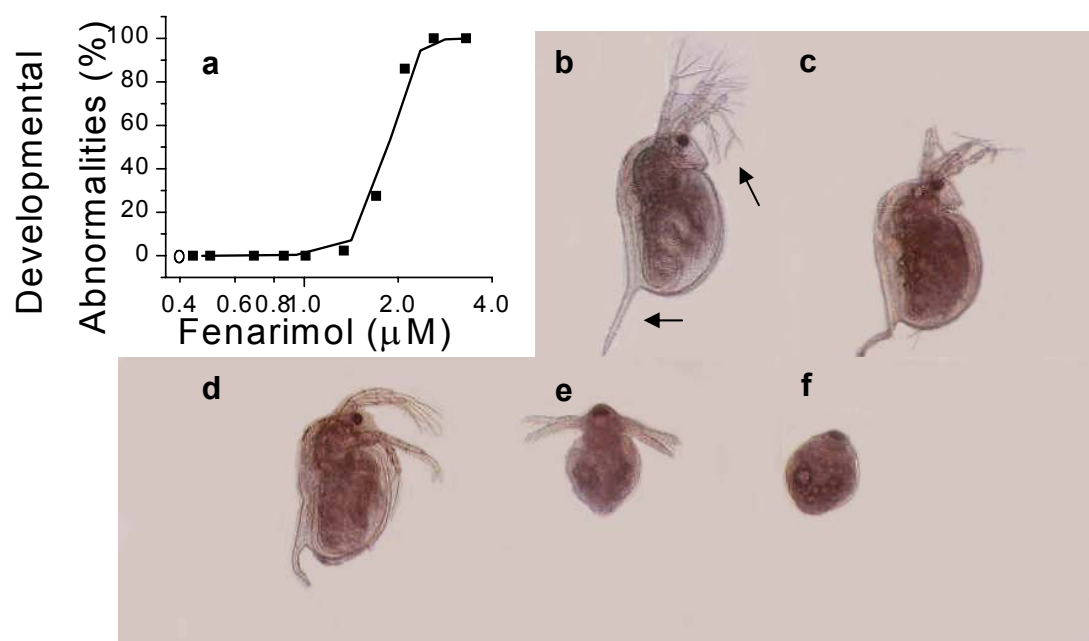


Figure 3

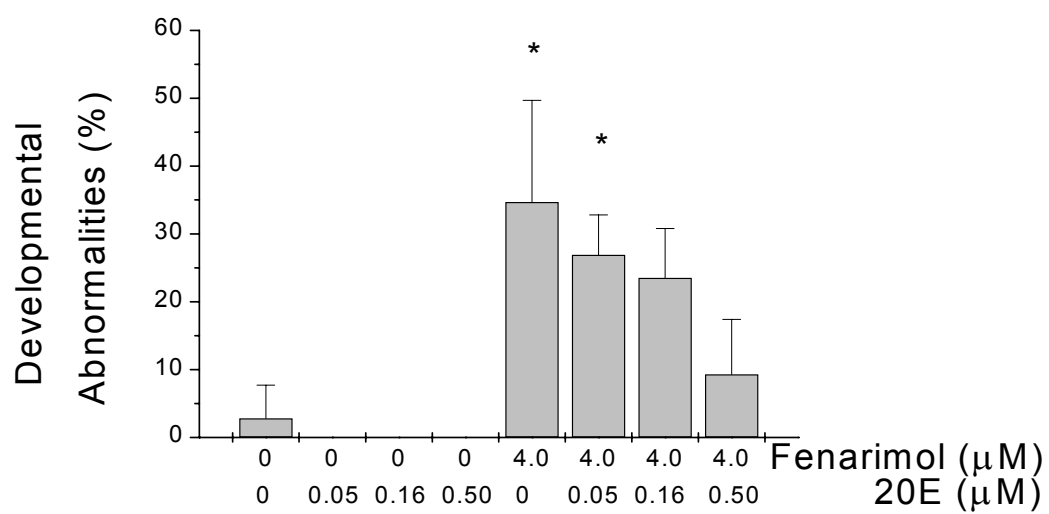


Figure 4

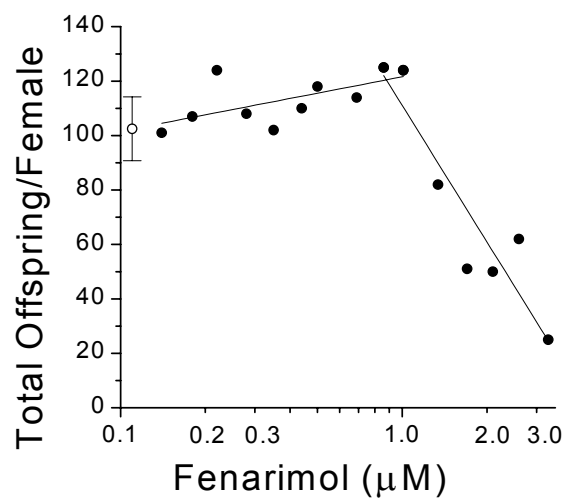


Figure 5

**Developmental Toxicity of Testosterone in the Crustacean
Daphnia magna Involves Anti-Ecdysteroidal Activity**

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Abstract

Testosterone has been shown to cause developmental arrest of embryonic daphnids (*Daphnia magna*). The present study was undertaken to determine whether this toxicity might be due to anti-ecdysteroidal activity associated with testosterone. The effect of testosterone on molt frequency of early instar daphnids was first evaluated to determine whether testosterone interfered with this ecdysteroid-regulated process. Molt frequency was delayed by exposure to testosterone and this effect was mitigated by co-exposure to the ecdysteroid 20-hydroxyecdysone. Testosterone exposure concentrations that interfered with molting also elicited developmental abnormalities among neonatal organisms produced by maternal organisms that were continuously exposed to testosterone or among embryos that were removed from unexposed mothers and exposed directly to the hormone. Embryos were significantly protected against the developmental toxicity of testosterone by co-exposure to 20-hydroxyecdysone. Take together, these results demonstrated that the embryo toxicity of testosterone to daphnids is due largely to its ability to interfere with ecdysteroid control of development. Experiments next were conducted to determine whether testosterone interfered with ecdysteroidal activity by acting as an ecdysone receptor antagonist or by reducing endogenous ecdysone levels. Testosterone significantly antagonized the action of 20-hydroxyecdysone in an ecdysone-responsive cell line. Testosterone had no discernable effect on endogenous ecdysone levels in daphnids. These results demonstrated that: 1) ecdysteroids regulate critical processes in daphnid embryo development, 2) testosterone elicits embryo toxicity to daphnids by interfering with ecdysteroid activity, and 3) ecdysteroid receptor antagonism could be one mechanism by which testosterone elicits these effects.

Introduction

The androgenic gland of malacostracan crustaceans produces androgenic hormone, a peptide hormone (Martin *et al.*, 1990) that appears to be primarily responsible for the development of the male reproductive organs in these organisms (Katakura, 1989; Suzuki *et al.*, 1989). In addition to androgenic hormone, the hormones 5-hydroxytryptamine and testosterone also have been shown to elicit a variety of masculinizing responses in crustaceans. 5-Hydroxytryptamine was shown to stimulate development of the androgenic gland and induce testicular maturation in immature male crayfish (Sarojini *et al.*, 1994). Similarly, testosterone has been shown to stimulate hypertrophy and hyperplasia of the androgenic gland of penaeid prawns (Nagabhushanam *et al.*, 1981), increase testis size in the prawn (Nagabhushanam *et al.*, 1981), convert ovaries to testes in the ocy pod crab (Sarojini, 1963), and mobilize glycogen from the midgut to the testis of the prawn (Nagabhushanam *et al.*, 1981). 5-Hydroxytryptamine was postulated to masculinize crayfish secondary to its stimulatory effects on the androgenic gland (Sarojini *et al.*, 1994). Masculinization of crustaceans by testosterone also could be secondary to its stimulatory effects on the androgenic gland.

We have observed that testosterone causes developmental arrest in embryos of the cladoceran *Daphnia magna* at exposure concentrations well below those that are overtly toxic to the maternal organisms (LeBlanc *et al.*, 2000). Furthermore, environmental chemicals that were shown to interfere with the metabolic clearance of testosterone by daphnids (i.e., propiconazole (Kast-Hutcheson *et al.*, 2001), 4-nonylphenol (LeBlanc *et al.*, 2000)) also

interfered with embryo development. These observations led us to hypothesize that, in all of these instances, embryo toxicity was the consequence of elevated testosterone levels (LeBlanc *et al.*, 1999).

Recently, we have reported that the agricultural fungicide fenarimol elicits similar effects on daphnid embryo development (Mu and LeBlanc, 2002). However, fenarimol elicited this effect by lowering endogenous ecdysone levels. Co-administration of 20-hydroxyecdysone was shown to protect daphnids against the embryo toxicity of fenarimol. Considering that fenarimol elicits embryo toxicity similar to testosterone, and that this toxicity of fenarimol is due to anti-ecdysteroidal activity of the fungicide, we considered the possibility that testosterone also may function as an anti-ecdysteroid in crustaceans. Ecdysteroids have been shown to exert feminizing or demasculinizing effects in male Diptera (summarized in (De Loof *et al.*, 1998)). Should ecdysteroids exert similar effects in crustaceans and should testosterone prove to have anti-ecdysteroidal activity, then the masculinizing effects of testosterone reported in prawns and crabs could be due to the anti-ecdysteroidal activity of this steroid compound.

In the present study we tested the hypothesis that the adverse effects of testosterone on the developing daphnid embryo are due to anti-ecdysteroidal properties of this compound. This hypothesis was tested first by evaluating the relationships between testosterone exposure levels and two ecdysteroid-regulated processes in daphnids– molting and embryo development. Secondly, the involvement of anti-ecdysteroidal activity of testosterone on

molting and embryo development was evaluated by determining the ability of exogenously administered 20-hydroxyecdysone to protect against the toxicity of testosterone. Lastly, the mechanism by which testosterone elicits anti-ecdysteroidal activity was determined.

Materials and Methods

Daphnia magna Daphnids were cultured and experimentally maintained in deionized water reconstituted with 192 mg/L CaSO₄·H₂O, 192 mg/L NaHCO₃, 120 mg/L MgSO₄, 8.0 mg/L KCl, 1.0 mg/L selenium and 1.0 mg/L vitamin B₁₂. Cultures were maintained at a density of 40 brood daphnids/L culture medium. Culture medium was renewed and offspring were discarded three times weekly. Brood daphnids were discarded after 3 weeks in the culture and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1.0 mL (~4 mg dry weight) of Tetrafin[®] fish food suspension (Pet International, Chesterfill, New South Wales, Australia) and 2.0 mL (1.4×10^8 cells) of a suspension of unicellular green algae, *Selenastrum capricornutum*. The algae were cultured in Bold's basal medium. Culture and experimental solutions were maintained at 20°C under a 16-hr photoperiod. These culture conditions maintained the daphnids in the parthenogenic reproductive phase.

Embryotoxicity of vertebrate-type steroid hormones Experiments were performed to determine whether testosterone is unique in its ability to interfere with embryo development of daphnids or whether this toxicity is a general effect of steroid compounds. Individual female daphnids (<24 hr old) were exposed to 8.0 µM cortisone, estradiol, androstenedione, and testosterone (Sigma, St. Louis, MO) in 50-mL beakers containing 40 mL of culture

medium. Each treatment consisted of 10 beakers, each containing a single daphnid. All treatments, including controls, contained the same amount (0.01%) of vehicle (absolute ethanol, AAPER, Shelbyville, KY) used to dissolve the steroids. Daphnids were provided food twice daily (Parks and LeBlanc, 1996), and test solutions were renewed three times weekly. Daphnids began releasing broods of parthenogenically-reproduced offspring on approximately day 7, each beaker was examined daily thereafter for released offspring. Offspring were counted, removed from the beakers, and examined microscopically to establish survival (heartbeat) and to identify any developmental abnormalities. Experiments were terminated after 21 days of exposure. Similar experiments also were performed where individual female daphnids were exposed to each of 20 concentrations of testosterone, with each concentration being 90% of the next highest concentration.

Effects of testosterone on molting and protection by 20-hydroxyecdysone Individual neonatal (<1 hour old) daphnids were exposed to each of 20 concentrations of testosterone, with each concentration being 90% of the next highest concentration or to a single concentration of testosterone along with increasing concentrations of 20-hydroxyecdysone (Sigma, St. Louis, MO) in 50-mL beakers containing 40 mL of medium. The effects of the steroids on molting were evaluated by measuring the duration of the first intermolt period to the nearest hour. Testosterone and 20-hydroxyecdysone solutions were prepared in absolute ethanol and all exposure solutions, including controls, contained 0.01% ethanol.

Embryotoxicity of testosterone and protection by 20-hydroxyecdysone Embryos that were in early stages of development (developmental stage 1 as defined previously (Kast-Hutcheson *et al.*, 2001)), were removed from untreated daphnids as described previously (LeBlanc *et al.*, 2000). Embryos were individually and randomly assigned to wells of 96-well microtiter plates along with 200 μ L of medium containing 12 μ M of testosterone and increasing concentration of 20-hydroxyecdysone. A concentration of 12 μ M testosterone was used since this concentration consistently produced a significant level of developmental abnormalities among exposed embryos (i.e., LeBlanc *et al.* 2000). All embryos, including controls, were exposed to the same concentration of ethanol vehicle (0.01%). Embryos were incubated at 20⁰C under a 16 hr photoperiod and were examined microscopically every 24 hr. Embryos were scored for stage of development and any abnormalities were noted. The percentage of embryos that exhibited developmental abnormalities was determined at the time at which development of the control daphnids was complete.

Anti-ecdysteroid activity analyses Ecdysone-responsive *Drosophila* Kc cells (kindly provided by Drs. Lucy and Peter Cherbas, Indiana University, Bloomington) were cultured in Schneider's medium (Sigma, St. Louis, MO) with 5% fetal calf serum (Sigma, St. Louis, MO) as previously described (Cherbas and Cherbas, 1998). In the absence of ecdysteroids, these cells are spherical shaped and readily proliferate. In the presence of ecdysteroids, these cells cease to proliferate and become spindle-shaped, elongate, and emit long pseudopodia (Cherbas *et al.*, 1989). Cells were plated in 96-well dishes at a density of $3-4 \times 10^5$ cells/mL. Cells were cultured in 0.10 μ M 20-hydroxyecdysone alone or 20-hydroxyecdysone with

increasing concentrations of testosterone. All exposure solutions, including controls, contained 0.5% ethanol. Cells were harvested after 3 days and counted using a hemocytometer. Cells also were examined for morphological transformation based upon their appearance as spindle-shaped or elongated and the percentage of transformed cells was determined.

Ecdysone radioimmunoassay Ecdysone levels in whole daphnids that were exposed to testosterone during the first intermolt period were measured by radioimmunoassay (Chang and O'Connor, 1979; Warren and Gilbert, 1988) as described previously (Mu and LeBlanc, 2002). The ecdysone antiserum was produced by Dr. W.E. Bollenbacher (University of North Carolina, Chapel Hill, NC) and distributed by Dr. E.S. Chang (University of California, Bodega Marine Laboratory, Bodega Bay, CA).

Statistics Statistical significance was evaluated by Analysis of Variance and Dunnett's t-test when comparing multiple treatments to the control and by Student's t-test when comparing a single treatment to the control using JMP software (SAS Institute, Cary, NC). The homogeneity of variances of the data was confirmed by Bartlett's test. Concentration–response curves were generated by linear regression or sigmoidal fit using Origin software (Microcal Software Inc., Northampton, MA).

Results

Embryotoxicity of vertebrate-type steroid hormones

Experiments were performed to determine whether the embryotoxicity of testosterone observed previously was common to other vertebrate-type steroid hormones. Daphnids were exposed to 8.0 μ M cortisone, estradiol, androstenedione, and testosterone. Effects on maternal survival, fecundity, and embryo development were assessed. All steroids had no significant effect on parental survival (Table 1). Cortisone, estradiol, and androstenedione had no significant effect on fecundity or development of F1 embryos. Testosterone significantly reduced the number of viable offspring produced and caused a significant incidence of developmental abnormalities among the offspring. Developmental effects ranged from minor abnormalities of the carapace and antennae to severely developmentally-arrested individuals. Thus, embryotoxicity associated with testosterone is not a characteristic shared by vertebrate-type steroid hormones in general.

Effects of testosterone on molting and protection by 20-hydroxyecdysone

Experiments next were performed to determine whether testosterone exhibits the characteristic anti-ecdysteroidal activity of delaying molting and whether this effect could be mitigated with exogenous 20-hydroxyecdysone. Testosterone delayed molting of daphnids, as measured by the duration of the first intermolt period (Fig. 1). The concentration-response line that described the relationship between testosterone concentration and intermolt duration exhibited a significant slope ($r=0.8327$, $p<0.0001$). Exposure of daphnids to 20-hydroxyecdysone, alone, had no significant effect on the time required for the organisms to molt at the concentrations evaluated (Mu and LeBlanc, 2002). However, exposure to

increasing concentrations of 20-hydroxyecdysone progressively abrogated the delay in molting caused by testosterone (Fig. 2).

Embryotoxicity of testosterone and protection by 20-hydroxyecdysone

Having established that testosterone does indeed elicit antiecdysteroid activity in a crustacean, we next evaluated the implications of this activity to embryo development. Exposure of maternal daphnids to testosterone caused a concentration-dependent increase in developmental abnormalities in offspring (Fig. 3a). Developmental effects ranged from minor abnormalities of the carapace and antennae to severely developmentally-arrested individuals. Commensurate with this occurrence of developmental abnormalities, progressively fewer viable offspring were produced with increasing testosterone concentration (Fig. 3b).

The mechanism by which testosterone interfered with normal development was investigated using daphnid embryos cultured in vitro (LeBlanc *et al.*, 2000). Direct incubation of daphnid embryos with 12 μM testosterone resulted developmental abnormalities that were consistent with those observed during maternal exposure. These developmental abnormalities were progressively mitigated by co-exposure to increasing concentrations of 20-hydroxyecdysone (Fig. 4). Exposure of embryos to these concentrations of 20-hydroxyecdysone alone has been shown to have no discernable effect on embryo development at the concentrations evaluated (Mu and LeBlanc, 2002). While co-treatment with 0.50 μM 20-hydroxyecdysone completely protected daphnids against the effects that 25 μM testosterone had on molting (Fig. 2), it only

partially protected daphnid embryos against the effects of 12 μ M testosterone. This reduced ability of 20-hydroxyecdysone to protect against developmental toxicity suggests that testosterone may have elicited toxicity to the embryos by ecdysone-independent mechanisms. These results indicated that the developmental toxicity associated with testosterone is, in part, a consequence of anti-ecdysteroid activity.

Mechanism of anti-ecdysteroid activity of testosterone

We are not aware of any ecdysone-responsive crustacean cell lines. Therefore, experiments were performed with ecdysone-responsive *Drosophila* Kc cells to determine whether testosterone developmental toxicity is elicited by competing with 20-hydroxyecdysone for occupancy of the ecdysone receptor. Proliferation of the cells was reduced and transformation increased with increasing concentration of 20-hydroxyecdysone (Fig. 5). Testosterone significantly antagonized this action of 20-hydroxyecdysone in the cells (Fig. 6). Exposure to increasing concentrations of testosterone progressively abrogated the suppressed proliferation and transformation caused by 20-hydroxyecdysone in the cells. However, cell proliferation was not completely restored at the testosterone concentrations evaluated, perhaps due to toxicity of the steroid.

Ecdysone levels were measured in testosterone exposed daphnids to determine whether the antiecdysteroidal activity of this compound was due, in part, to suppression of endogenous ecdysteroid levels. Testosterone had no discernable effect on endogenous ecdysone levels in daphnids that were exposed through the first intermolt period (Table 2).

Discussion

The purpose of this study was to test the hypothesis that testosterone functions as a developmental toxicant in daphnids by acting as an anti-ecdysteroid and interfering with ecdysteroid-regulated processes. Results demonstrate that, in addition to interfering with daphnid embryo development, testosterone delayed molting of neonatal organisms – a well-characterized ecdysteroid-regulated process. These effects were shown to be due, at least in part, to antiecdysteroidal activity of testosterone, since provision of exogenous ecdysteroid (20-hydroxyecdysone) protected daphnids against these effects of testosterone. Taken together, these results warrant acceptance of the hypothesis regarding the mechanism of developmental toxicity of testosterone.

The embryo toxicity that is characteristic of testosterone, was not shared by other steroid hormones, at equimolar exposure concentrations. The incidence of developmental abnormalities among embryos exposed to androstenedione was greater than that observed among control daphnids, though the incidence was not statistically significant. We have previously observed that androstenedione causes developmental abnormalities in daphnid embryos in a concentration-dependent manner (LeBlanc, 1999) though androstenedione appears to be a less potent developmental toxicant as compared to testosterone. This characteristic thus may be generally associated with vertebrate-type steroidal androgens.

Finally, we demonstrate in this study that the anti-ecdysteroidal activity of testosterone could be due to its ability to compete with ecdysteroids for occupancy of the ecdysteroid receptor. Testosterone, itself, exhibited no ecdysteroidal activity in the ecdysone responsive cells used in this study. However, testosterone was able to block the activity of 20-hydroxyecdysone when both steroids were provided to the cells. Since we did not directly measure binding of testosterone to the ecdysone receptor, other interpretations of these experiments must be considered. For example, testosterone may have induced expression of enzymes that facilitated the metabolism of ecdysone in the cultured cells. We previously demonstrated that exposure to 20-hydroxyecdysone significantly increased the rate of elimination of testosterone as sulfate conjugates in daphnids (Baldwin and LeBlanc, 1994). Perhaps, testosterone similarly enhances the metabolism of ecdysteroids. However, ecdysteroid levels in testosterone-treated daphnids were not altered in these experiments (Table 2) which argues against some effect of testosterone on ecdysone metabolism.

Dinan *et al.* (2001) evaluated the ecdysteroidal antagonistic activity of androstenedione and testosterone using *Drosophila* B₁₁ cells and methods similar to those used in the present study. They reported that androstenedione elicited weak antagonistic activity at a concentration of 100 μ M and testosterone did not elicit antagonistic activity at 10 μ M, the highest concentration evaluated. These results are consistent with the present study where androstenedione exhibited evidence of weak antiecdysteroidal activity in vivo (Table 1). Using cultured cells, testosterone did not elicit antiecdysteroidal activity at 10 μ M but elicited significant antiecdysteroidal activity at 100 μ M.

We previously demonstrated that the agricultural fungicide fenarimol elicited antiecdysteroidal activity by reducing endogenous ecdysone levels in daphnids (Mu and LeBlanc 2002). In the present study, we demonstrate that testosterone also elicits anti-ecdysteroidal activity in daphnids, albeit through a different mechanism. A comparison of the responses of daphnids to these two antiecdysteroids may provide insight that can be used to garner mechanistic information on other antiecdysteroidal chemicals. For example, in other species thusfar examined, embryonic ecdysteroids originate from two sources (Subramoniam, 2000). Ecdysteroids in the early embryo originate from the mother and are transferred to the egg. Ecdysteroids present in the late embryo are derived, at least in part, from de novo synthesis of the hormone by the embryo. Thus, exposure of isolated embryos (presented in Fig. 4) to an ecdysteroid receptor antagonist would be predicted to affect the activity of both pools of ecdysteroid, perhaps resulting in both early and late developmental abnormalities. In contrast, direct exposure of embryos to a compound that inhibits ecdysteroid synthesis (i.e., fenarimol) would be predicted to have no effect on the existing maternal pool of ecdysteroid in the embryo and thus may elicit primarily late developmental abnormalities. Studies are underway to test this potential diagnostic aspect of the embryo assay.

Concern for the adverse effects of environmental endocrine toxicants has increased precipitously over the past decade and has culminated, in the United States, with legislation

that will require evaluation of chemicals of toxicity towards estrogen, androgen, and thyroid regulated processes. Clearly, these hormones are important to vertebrates and they are susceptible to disruption by environmental chemicals. However, the role of these hormones in invertebrate endocrinology is less evident (LeBlanc *et al.*, 1999). As demonstrated in the present study, testosterone does perturb endocrine function in daphnids resulting in developmental toxicity but this effect is not due to androgenicity. Rather, testosterone is functioning as an anti-ecdysteroid, a mechanism of high relevance to arthropods but of little significance to vertebrates. Thus, when considering the endocrine toxicity of environmental chemicals to invertebrates, it is critical that the susceptibility of invertebrate species be directly evaluated. There is little basis for the extrapolation of experimental indications of endocrine disruption derived from experiments with vertebrate species to invertebrates.

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Table 1. Performance of daphnids exposed to 8.0 μ M of vertebrate-type steroid hormones for 3 weeks. Data are presented as mean and SEM (n=10). An asterisk indicates a significant difference from the control at $P \leq 0.05$ (ANOVA, Dunnett's t test).

Treatment	Parental Survival (%)	Offspring/ Female	Abnormality (%)
Control	90	76.8 $\pm$ 3.7	0.6 $\pm$ 0.6
Cortisone	80	82.1 $\pm$ 9.5	0.8 $\pm$ 0.3
Estradiol	90	83.5 $\pm$ 9.8	1.0 $\pm$ 0.7
Androstenedione	100	87.3 $\pm$ 4.0	6.1 $\pm$ 1.7
Testosterone	90	49.0 $\pm$ 5.2*	66.9 $\pm$ 6.0*

Table 2. Ecdysone levels in daphnids that were exposed to 25 μ M testosterone during the first intermolt period. Data are presented as the mean and SEM (n = 13 groups in control, and n = 10 groups in treatment, each group containing 25-45 individual organisms).

Treatment	Ecdysone (pg/neonate)
Control	21.0 $\pm$ 6.5
Testosterone	28.6 $\pm$ 7.2

Figure legends

Figure 1. Influence of testosterone on molting as measured by the duration of the first intermolt period. The dotted line represents the mean duration of the intermolt period of 10 control daphnids.

Figure 2. Influence of 20-hydroxyecdysone (20E) on the testosterone-induced increase in the duration of the first intermolt period. Neonatal daphnids (<1 hour old) were individually exposed to 25 μ M testosterone alone or in combination with concentrations of 20E. Data are presented as mean and SEM (n=10). An asterisk denotes a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's t test).

Figure 3. Chronic toxicity resulting from maternal testosterone exposure. **a:** Incidence of developmental abnormalities resulting from maternal exposure to concentrations of testosterone. **b:** Total offspring produced by maternal daphnids during exposure to concentrations of testosterone. Each data point represents the total number of offspring produced by a single parthenogenically reproducing female over a 21-day exposure period. Performance of control daphnids (mean $\pm$ SEM, n=10) is depicted as the open circle with error bars.

Figure 4. Mitigation of testosterone-induced developmental abnormalities by 20-hydroxyecdysone (20E) during in vitro embryo exposure. Data are presented as the mean $\pm$

SEM (n=3 groups per treatment. Each group consisted of 60-85 embryos). An asterisk denotes a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's t test). A # denotes a significant difference from the testosterone treatment alone at $p \leq 0.05$ (ANOVA, Dunnett's t test).

Figure 5. Concentration-response effects of 20-hydroxyecdysone in Kc cells on **(a)** cell proliferation, and **(b)** cell morphological response. Data are presented as the mean $\pm$ SEM (n=3 groups per treatment). An asterisk denotes a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's t test).

Figure 6. Antagonistic activity of testosterone on the action of 20-hydroxyecdysone (20E) in Kc cells. **a:** Mitigation of 20E-induced cell proliferation commitment by testosterone. **b:** Ablation of 20E-induced cell morphological transformation by testosterone. Data are presented as the mean $\pm$ SEM (n=3 groups per treatment). An asterisk denotes a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's t test). A # denotes a significant difference from the 20E treatment alone at $p \leq 0.05$ (ANOVA, Dunnett's t test).

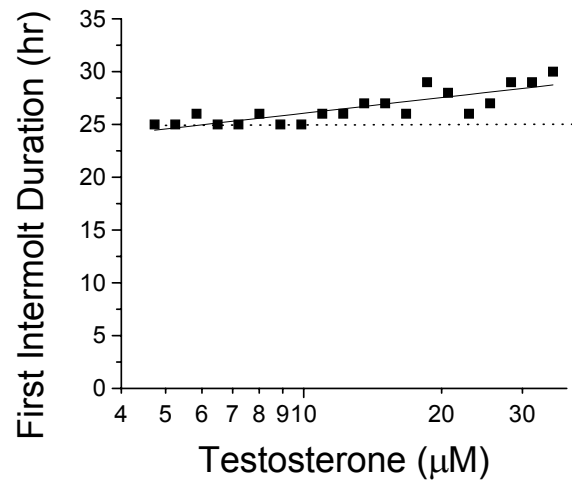


Figure 1

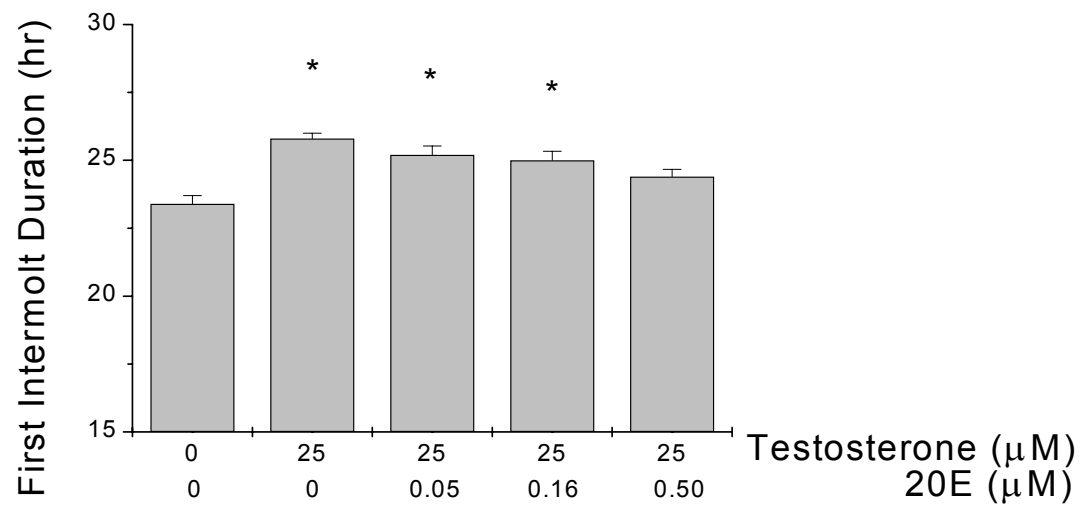


Figure 2

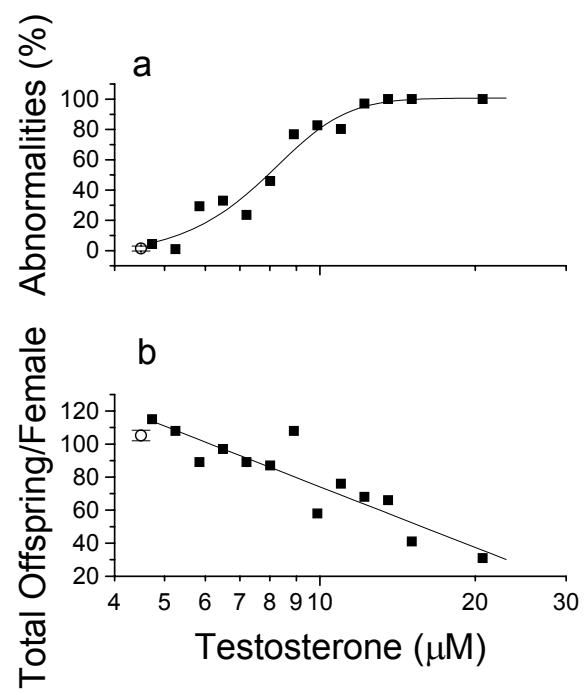


Figure 3

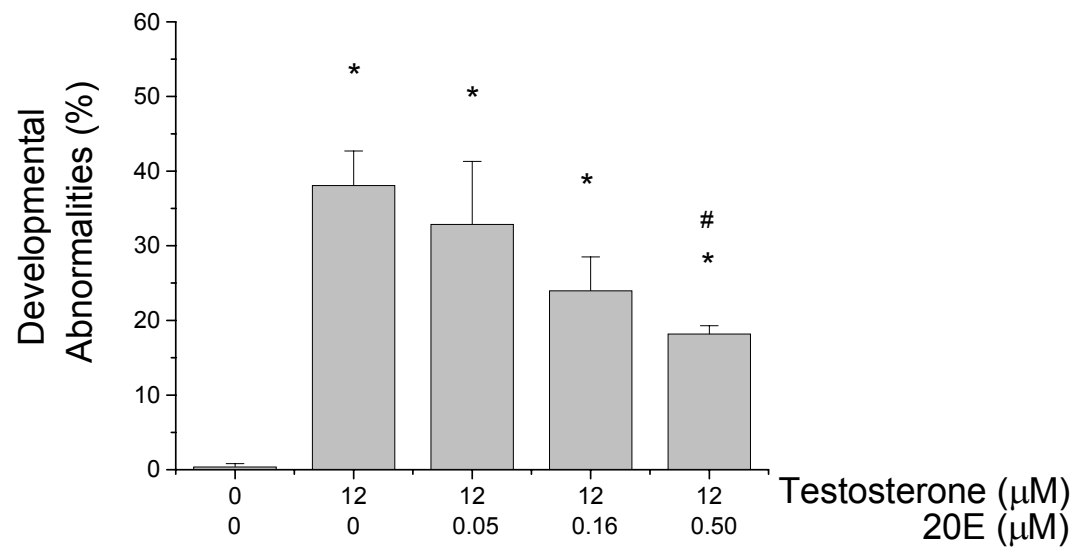


Figure 4

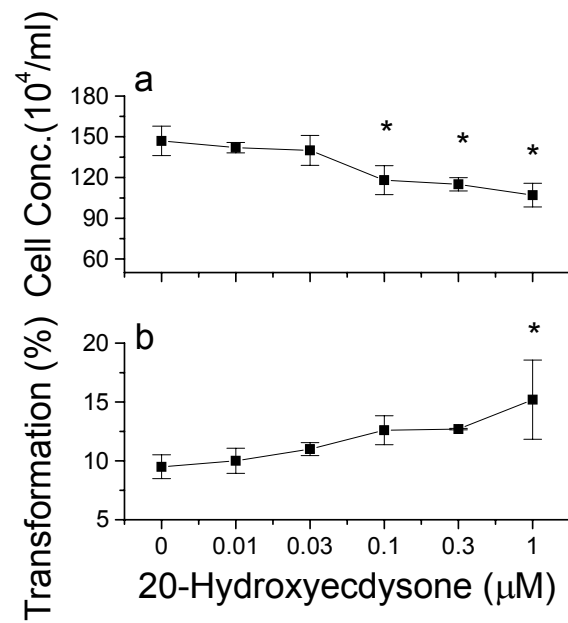


Figure 5

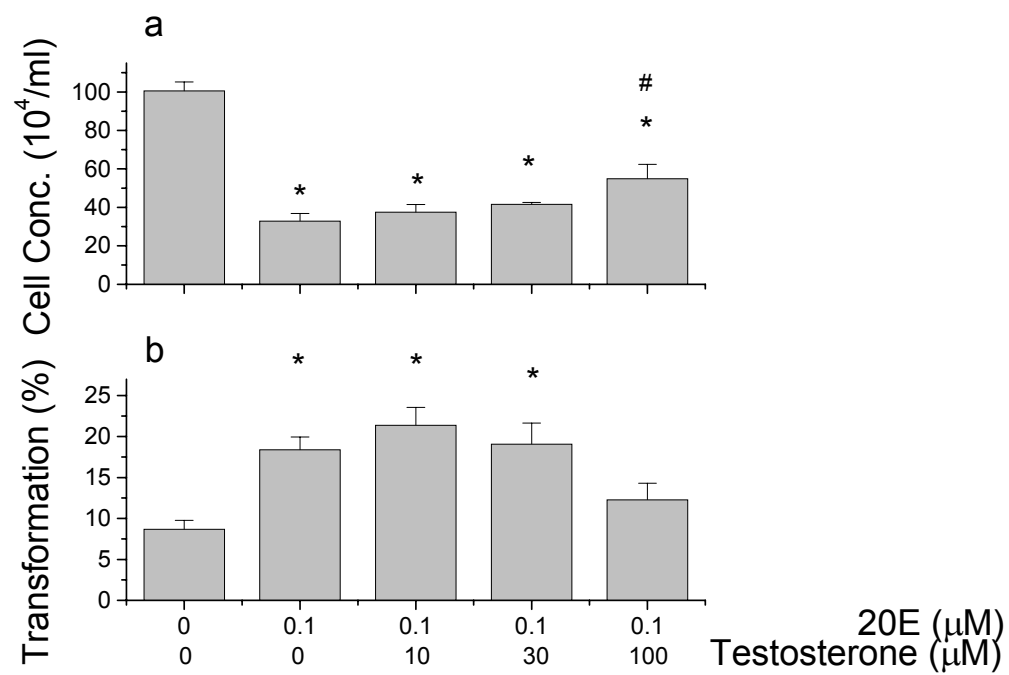


Figure 6

**Synergistic Interaction of Endocrine Disrupting Chemicals: Model
Development Using an Ecdysone Receptor Antagonist and a Hormone
Synthesis Inhibitor**

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Abstract

Endocrine toxicants can interfere with hormone signaling through various mechanisms. Some of these mechanisms are interrelated in a manner that might result in synergistic interactions. Here we tested the hypothesis that combined exposure to chemicals that inhibit hormone synthesis and that function as hormone receptor antagonists would result in greater than additive toxicity. This hypothesis was tested by assessing the effects of the ecdysteroid-synthesis inhibitor fenarimol and the ecdysteroid receptor antagonist testosterone on ecdysteroid-regulated development in the crustacean *Daphnia magna*. Both compounds were individually characterized for effects on the development of isolated embryos. Fenarimol caused late developmental abnormalities, consistent with its effect on offspring-derived ecdysone in the maturing embryo. Testosterone interfered with both early and late development of embryos, consistent with its ability to inhibit ecdysone provided by maternal transfer (responsible for early developmental events) or de novo ecdysone synthesis (responsible for late developmental events). We predicted that, by decreasing endogenous levels of hormone, fenarimol would enhance the likelihood of testosterone binding to and inhibiting the ecdysone receptor. Indeed, fenarimol enhanced the toxicity of testosterone; while, testosterone had no effect on the toxicity of fenarimol. Algorithms were developed to predict the toxicity of combinations of these two compounds based upon independent joint action (IJA) alone, as well as IJA with fenarimol-on-testosterone synergy (IJA+SYN). The IJA+SYN model was highly predictive of the experimentally-determined combined effects of

the two compounds. These results demonstrate that some endocrine toxicants can synergize, and this synergy can be accurately predicted.

Key words: endocrine, disruptors, invertebrate, mixtures, synergy

Introduction

Evaluating the toxicity of chemical mixtures is one of the most pertinent toxicological issues to face modern society and one of the most complex challenges to modern toxicology. Organisms are rarely exposed to individual chemicals in the environment. Recent revelations of the multiplicity of xenobiotics found in surface waters of the United States [1] and within the human body [2] have amply demonstrated the need to develop tools for use in predicting the hazard associated with chemical mixtures.

Endocrine toxicants may harbor an increased likelihood for non-additive interactions owing to their potential to elicit effects at different targets along a common signaling pathway. Most often, the consequences of such interactions will conform to a model for independent joint action where effects of different chemicals are elicited at independent targets though a common outcome occurs [3,4]. However, scenarios could arise whereby one endocrine toxicant modifies the potency of another resulting in either an antagonistic or synergistic effect. Indeed, synergistic effects of endocrine toxicants have been reported, though the mechanisms responsible for these non-additive interactions typically have remained elusive [5-7].

A common mode of toxic action of endocrine toxicants is hormone receptor antagonism [8]. Receptor antagonism typically involves competition for receptor occupancy between the xenobiotic and the endogenous hormone. The xenobiotic blocks hormone action

by occupying the receptor while eliciting little or no activation of the receptor [8]. Steroid hormone receptors, in general, seem to be susceptible to the antagonistic effects of xenobiotics [8-10]. Alternatively, some chemicals can elicit anti-hormonal effects by interfering with synthesis of the hormone. Inhibitors of cytochrome P450 enzymes have been shown to interfere with steroid hormone synthesis resulting in reduced hormone levels and associated endocrine toxicity [11-13].

Since steroid hormone receptor antagonists must compete with endogenous hormone for receptor occupancy, levels of the endogenous hormone will impact the efficacy of receptor binding by the antagonist. Binding of the antagonist to the receptor will increase with decreasing concentration of the competing endogenous hormone. Thus, in addition to eliciting independent actions on processes regulated by the targeted hormone, hormone synthesis inhibitors and receptor antagonists are predicted to synergize.

In this study, we tested the hypothesis that a hormone synthesis inhibitor and a hormone receptor antagonist would synergize to yield greater toxicity than that predicted from a model of independent joint action. This hypothesis was tested using isolated embryos from the crustacean *Daphnia magna*. Development of daphnid embryos is highly dependent upon ecdysteroids [13]. Effects of fenarimol, an ecdysteroid synthesis inhibitor [13,14] and testosterone, an ecdysteroid receptor antagonist [15] on ex vivo embryo development were evaluated independently and in combination in order to detect and quantify any synergistic interactions.

Ecdysteroids associated with the developing arthropod embryo are derived from two sources (summarized in [16]). Maternally derived ecdysteroids are packaged into the egg and typically regulate early aspects of embryo development. Following organogenesis, de novo synthesis of ecdysteroids occurs in the embryo. These ecdysteroids may contribute to the regulation of late aspects of embryo development. Accordingly, we hypothesized that treatment of isolated embryos with the ecdysteroid synthesis inhibitor fenarimol would result only in perturbations late in embryo development since maternally-derived ecdysteroids would be present and active in the embryo early in development. However, treatment of isolated embryos with the ecdysteroid receptor antagonist testosterone would be predicted to interfere with the action of ecdysteroid regardless of source resulting in both early and late developmental abnormalities. The different developmental abnormalities (early versus late) caused by fenarimol and testosterone were evaluated in the present study as a means of confirming the differential modes of antiecdysteroidal activity of the compounds.

Finally, mathematical models were developed based on the established modes of antiecdysteroidal activity and synergy of these two compounds and model predictions were tested experimentally. Experimental validation of the appropriate model would lend additional support to our proposed modes of action and interaction of these two compounds. Lastly, these results would demonstrate whether synergistic interactions between endocrine toxicants can be accurately predicted based upon a mechanistic understanding of the compounds involved.

Materials and Methods

Model organism: Daphnids (*Daphnia magna*) were cultured and maintained in experiments in formulated media [15]. Cultures were maintained at a density of 40 brood organisms/L culture medium. Culture medium was renewed and offspring were discarded three times weekly. Brood daphnids were discarded after 3 weeks in the culture and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1.0 mL (~4 mg dry weight) of Tetrafin[®] fish food suspension (Pet International, Chesterfill, New South Wales, Australia) and 2.0 mL (1.4×10^8 cells) of a suspension of unicellular green algae *Selenastrum capricornutum*. The algae were cultured in Bold's basal medium. Culture and experimental solutions were maintained at 20°C under a 16 hr photoperiod. Daphnids are capable of reproducing either sexually or asexually (parthenogenesis). Under these culture conditions daphnid populations consisted entirely of females that reproduced by parthenogenesis.

Ecdysone radioimmunoassay: Radioimmunoassays (RIA) were performed to measure immunodetectable ecdysone levels in juveniles and embryos during various stages of development. Ecdysone was extracted from whole daphnids and quantified by RIA as described [13]. The ecdysone antiserum was produced by Dr. W.E. Bollenbacher (University of North Carolina, Chapel Hill, NC) and distributed by Dr. E.S. Chang (University of California, Bodega Marine Laboratory, Bodega Bay, CA).

Developmental toxicity of antiecdysteroids: Embryo toxicity of fenarimol and testosterone were individually evaluated in order to identify differences in age susceptibility of the embryos to these two antiecdysteroids. Embryos that were in early stages of development (developmental stage 1 as defined previously [17]), were removed from untreated maternal daphnids as described previously [18]. Embryos were individually and randomly assigned to wells of 96-well microtiter plates along with 200 μ L of medium containing 4.0 μ M of fenarimol (Chem Service, West Chester, PA) or 12 μ M of testosterone (Sigma, St. Louis, MO) dissolved in absolute ethanol (AAPER, Shelbyville, KY). All embryos, including controls, were exposed to the same concentration of ethanol vehicle (0.01%). Embryos were incubated at 20°C under a 16 hr photoperiod and were examined microscopically at 24 hr intervals for 72 hrs. Embryos were scored for stage of development, as described previously [17], and any abnormalities were noted and documented using a digital camera (Pixera Corporation, Los Garos, CA) affixed to the microscope. Developmental abnormalities were scored as early or late. Embryos that exhibited early developmental abnormalities typically did not progress beyond stage 4. Most notably, the second embryonic membrane that normally ruptures between stages 4 and 5 remained intact around the embryos. Embryos that exhibited late developmental abnormalities progressed to near complete development but exhibited abnormally curved shell spines and underdeveloped second antennae. Significant differences in the incidence of developmental abnormalities among treatment groups were evaluated by analysis of variance (ANOVA) and Dunnett's t-test following arcsin transformation of the data (JMP software, SAS institute, Cary, NC).

Antiecdysteroid interactions: Potential interactive effects of the antiecdysteroids on ecdysteroid-dependent embryo development were modeled based upon the previously demonstrated modes of action of the chemicals and the observed effects of the individual materials. Model predictions then were assessed experimentally by evaluating the effects of defined binary combinations of the materials on embryo development and comparing the observed results to the model predictions.

Model formulation Results from the present study indicated that the two chemicals elicited the same response, characteristic of antiecdysteroidal activity, but through different mechanisms; therefore, an independent joint action model of additivity [3] was selected as the foundation model upon which the combined action of the chemicals would be described. This model defines the situation where the proportion of individuals responding to the chemical mixture is equal to the sum of the proportions of individuals that respond to each material. This model is defined by the equation:

$$R_{\text{mixture}} = R_t + R_f - (R_t)(R_f) \quad (1)$$

where R_{mixture} represents the proportion of embryos that respond to the mixture. R_t and R_f represent the proportions of embryos that respond to the testosterone and fenarimol, respectively. R_t and R_f were determined from direct evaluation of the effects of testosterone or fenarimol, at defined concentrations, on embryo development. Briefly, embryos were exposed to serial concentrations of testosterone and fenarimol that were predicted to elicit effects covering the range from 0 to 100% as described under the heading “Developmental toxicity of antiecdysteroids”. Concentration-response curves were used, following

linearization by log-probit transformation (Origin software, Microcal Software Inc., Northampton, MA) to interpolate the response (R) at a defined concentration of the chemical.

This model also was modified to incorporate the putative synergistic interaction between the chemicals. This model was based upon the assumption that both compounds would elicit independent effects on ecdysteroid-dependent processes, but in addition, fenarimol would increase the potency of testosterone by lowering the endogenous ecdysteroid levels with which testosterone competes for receptor occupancy. This putative effect of fenarimol on testosterone potency (termed “fenarimol-on-testosterone synergy”) was evaluated by quantifying the effects of fenarimol on the concentration-response curve for testosterone. Experiments were performed using the ex vivo embryo exposure methods described previously. Embryos were exposed to serial concentrations of testosterone in the presence of subtoxic levels of fenarimol. Subtoxic levels were defined as being equal to or less than the no-observed-effect-level (NOEL) that was derived from the concentration-response experiments. EC₅₀ values were calculated by probit analysis (PROC PROBIT, SAS 8.0 software, SAS institute, Cary, NC). The linear relationship was determined between the EC₅₀ values derived for testosterone in the presence of increasing subtoxic concentrations of fenarimol. This relationship was used to define the ‘coefficient of interaction’ or K value [19]:

$$K_x = (EC_{50_0}) / (EC_{50_x}) \quad (2)$$

where K_x is the coefficient of interaction at fenarimol concentration 'x', EC_{50_0} is the EC_{50} of testosterone in the absence of fenarimol, and EC_{50_x} is the EC_{50} of testosterone in the presence of concentration 'x' of fenarimol.

The K value represented the degree to which the modifying chemical (fenarimol), at a defined exposure concentration ('x'), shifted the concentration-response curve for the primary chemical (testosterone). K values were incorporated into the independent joint action model as follows:

$$C_t' = C_t(K) \quad (3)$$

where C_t is the concentration of testosterone present in the combination; K represents the coefficient of interaction for the concentration of fenarimol in the combination; and C_t' represents the adjusted concentration of testosterone in the combination as modified by fenarimol. A modified model for independent joint action then was used to calculate the toxicity of the mixture:

$$R_{\text{mixture}} = R_t' + R_f - (R_t')(R_f). \quad (4)$$

where R_t' represents the response to the modified concentration of testosterone. All other model components are as described for equation 1. The model describes both the independent actions of testosterone and fenarimol, as well as the modifying effect of fenarimol on the endocrine toxicity of testosterone. Similar experiments were performed to evaluate any effect of testosterone on the toxicity of fenarimol in order to calculate K values that describe testosterone-on-fenarimol synergy, should it occur.

Model Assessment Effects of the chemical mixtures on embryo development were determined using binary combinations of the materials. Five concentrations of testosterone (1.5, 3.0, 4.5, 6.0, 9.0 μM) were used in combination with serial concentrations of fenarimol (0.5, 0.7, 0.9, 1.1, 1.3, 1.7, 2.1, 2.6, 3.3, 4.1, 5.1 μM), that were predicted by the independent joint action model to elicit effects covering the range from 0 to 100%. A total of thirty combinations of testosterone and fenarimol were evaluated for combined effects on embryo development as described under the heading “Developmental toxicity of antiecdysteroids” and results were compared to the models for independent joint action (equation 1) and independent joint action with fenarimol-on-testosterone synergy (equation 4). Experimental (observed) results were compared to predicted (modeled) results using correlation analyses. The model which best predicted the observed results was judged by the greater coefficient of determination (r^2) and greater concordance to a one-to-one relationship as defined by the linear association [20]. Linear associations with 95% confidence intervals, and coefficients of determination were calculated using Origin software (Microcal Software Inc., Northampton, MA).

Results

Ecdysone levels in embryos

An understanding of the effects of the antiecdysteroids on embryo development first required an evaluation of the temporal changes in the hormone levels in the developing embryo. Periods of elevated hormone levels would be indicative of windows of embryo

development that are regulated by ecdysteroids. Immunodetectable ecdysone levels were high early in embryo development and progressively declined through stage 4 (Fig. 1). Beyond stage 4, ecdysone levels slightly increased. These changes were consistent when ecdysone levels were normalized either to the number of embryos analyzed (Fig. 1A) or to the wet tissue weight of the embryos (Fig. 1B). These results suggest the existence of two pools of ecdysteroid in the daphnid embryo. A pool is present in the early embryo that is likely of maternal origin. This pool is largely exhausted by stage 4, but then is supplemented by ecdysteroid that may result from de novo synthesis. The immunoassay used was verified with juvenile daphnids (Fig. 2). Immunodetectable ecdysone levels were low (generally below 50 pg/mg) through most of the molt cycle of the organism but transiently increased to approximately 150 pg/mg just prior to molting. This profile of ecdysone levels is consistent with that measured through the molt cycle of other crustaceans [21-24].

Differential developmental toxicity of fenarimol and testosterone

Experiments next were conducted to determine whether the ecdysteroid synthesis inhibitor fenarimol and the ecdysteroid receptor antagonist testosterone elicited differential effects on isolated developing embryos. Direct exposure of embryos to fenarimol interfered primarily with late events in embryonic development (Fig. 3). Fenarimol-associated abnormalities consisted essentially of poorly developed second antennae and curved or unextended shell spines (Fig. 4 C,D). Direct exposure of isolated embryos to testosterone interfered with both early and late events in embryo development (Fig. 3). Effects of testosterone on early development were characterized by apparent developmental arrest at or

prior to stage 4 (Fig. 4B). These results are consistent with the postulate that the early ecdysteroid pool is of maternal origin and its activity in the embryo is susceptible to the action of ecdysteroid receptor antagonists, but not ecdysone synthesis inhibitors. While, the late pool of ecdysteroid is derived from de novo synthesis in the embryo and its activity is susceptible to both ecdysteroid receptor antagonists and ecdysteroid synthesis inhibitors.

Chemical interactions

The differential response of daphnid embryos to the toxicity of fenarimol and testosterone corroborated previous observations that these two materials elicit antiecdysteroidal activity through two different mechanisms. These observations allowed us to hypothesize that combinations of these materials would elicit toxicity that conformed, at least in part, to the model for ‘independent joint action’ (IJA). According to this model, both fenarimol and testosterone would elicit the same endocrine effect (developmental toxicity) to embryos, but through two distinct mechanisms. This hypothesis was tested by experimentally determining the embryo toxicity of combinations of the materials and comparing results to model predictions. Isolated daphnid embryos were exposed to thirty combinations of fenarimol and testosterone that were predicted to affect from 0 to 100% of the exposed embryos using the model for independent joint action (equation 1) and concentration-response data generated with the individual compounds (Fig. 5).

The IJA model alone poorly predicted the observed combined effects of the two antiecdysteroids (Fig. 6A). The model commonly underestimated toxicity of the

combinations. Variability between observed and modeled results was high ($r^2=0.48$) and the line that defined a one-to-one relationship between observed and modeled results ($Y=0+1(X)$) typically existed outside of the 95% confidence interval for the derived linear association. These results implied that a synergistic interaction existed between the two chemicals. The prior evaluation of the mechanisms of toxicity of these materials provided mechanistic support for this synergy. Therefore, we expanded the model to include the appropriate descriptor of synergism (K value).

The first objective in the evaluation of synergy was to measure the effect of fenarimol on the embryo toxicity of testosterone. Daphnid embryos were exposed to concentrations of testosterone that defined the concentration-response curve for this material in the presence of concentrations of fenarimol that were below the threshold concentration for this compound. These low levels of fenarimol were necessary to avoid any confounding effects due to direct developmental toxicity of the fenarimol. The embryo toxicity of testosterone increased with increasing fenarimol concentration (Table 1). Conversely, sub-threshold concentrations of testosterone had no effect on the toxicity of fenarimol (Table 1). These results confirmed that fenarimol enhances the toxicity of testosterone and this synergistic interaction must be considered when modeling the combined toxicity of these antiecdysteroids. Accordingly, we expanded our model of the combined effects of these compounds to ‘independent joint action with fenarimol-on-testosterone synergy’ (IJA+SYN).

The synergistic effect of fenarimol on testosterone developmental toxicity was quantified in terms of a K value. K values for various fenarimol exposure concentrations were calculated (Table 1) and plotted against fenarimol exposure concentrations. A linear relationship existed between these two parameters ($r=0.9978$). For modeling purposes, this relationship was extrapolated to higher fenarimol concentrations. K values could not be measured directly at these higher fenarimol concentrations because of direct developmental toxicity. K values were then derived from this line and incorporated into the IJA+SYN model (equations 2-4).

Adding the synergistic function to the IJA model dramatically improved the ability of the model to accurately and precisely described the toxicity of the fenarimol-testosterone combinations (Fig. 6B). Variability between observed and modeled results was appreciably reduced using this model ($r^2=0.86$) and the line that defined a one-to-one relationship between observed and modeled results ($Y=0+1(X)$) existed within the 95% confidence interval for the derived linear association.

Discussion

The hypothesis tested was that a hormone synthesis inhibitor and hormone receptor antagonist would synergize to yield greater toxicity than predicted from a model of independent joint action. Results support this hypothesis and define a discrete mechanism by which endocrine toxicants can synergize. Both hormone synthesis inhibitors and hormone

receptor antagonists can elicit a common response characteristic of anti-hormone activity. However, by lowering endogenous hormone levels, the synthesis inhibitor will increase the competitive advantage of the receptor antagonist for receptor binding and thus increase the activity of this compound.

This hypothesis was tested and the model for synergy developed using an *in vivo* system consisting of ecdysteroid-responsive development of crustacean embryos. Ecdysteroids do not function as endocrine signaling molecules in vertebrates. However, the ecdysteroid signaling pathway is structurally and functionally orthologous to steroid hormone regulatory pathways of vertebrates [25]. Compounds that inhibit P450-mediated ecdysteroid biosynthetic pathways in crustaceans are likely to inhibit steroid biosynthetic pathways in vertebrates [26]. Such is the case with the agricultural fungicide fenarimol used in this study. At exposure concentrations that elicit no overt toxicity to maternal daphnids, fenarimol significantly reduces ecdysone levels in developing embryos and interferes with ecdysone-dependent development of the embryo [13]. Receptor antagonism also is a common mechanism of endocrine toxicity to both invertebrates and vertebrates. A variety of structurally-diverse chemicals have been reported to antagonize vertebrate estrogen [27], androgen [9, 28], and progestogen [29] receptors. Thus, the mechanism of synergy described in this study using an invertebrate model would also be of relevance to vertebrates.

The predictive model developed was highly effective in describing the joint toxicity of various binary mixtures of the antiecdysteroids. This model incorporated both the

independent joint action of the two compounds as well as the synergistic effects of one compound on the other. Approaches to modeling synergistic interactions of chemicals have been available for decades (i.e.[19]). However, recent interests in chemical interactions have focused primarily on the detection of the interaction rather than its prediction [30,31]. This may be due to the relative dearth of mechanistic information that is necessary to predict interactions among chemicals. Increased research emphasis on defining mechanisms of interactions, both synergistic and antagonistic, between chemicals will greatly augment the ability to accurately predict the toxicological consequences of exposure to chemical mixtures.

The coefficient of interaction (K), used in the model, described the synergistic effect of a defined concentration of fenarimol on the embryo toxicity of testosterone. The valid use of these K values, which were derived from the shift in testosterone EC₅₀ values, is predicated on the assumption that the shape (or slope) of the testosterone concentration-response curves was unaffected by fenarimol treatment. Such an assumption is generally reasonable and was demonstrated to be valid in the present study (data not shown). A second assumption made in the use of K values was that the relationship between fenarimol concentration and magnitude of the K value remained constant at concentrations of fenarimol greater than those experimentally evaluated. K values could not be experimentally determined at high fenarimol concentrations since the fenarimol itself would cause embryo toxicity. It would be impossible to discern whether the additional embryo toxicity was due to synergy with testosterone or due to the direct effects of the fenarimol. The high level of

concordance between the observed effects of the binary mixtures at high fenarimol concentrations and the modeled outcomes indicates that this assumption was valid.

A crustacean model was used in this study to address some fundamental issues in the toxicology of chemical mixtures. However, results also may be directly relevant to the reported decline of some crustacean populations [32-35]. Many factors including weather, over harvesting, and loss of habitat have likely contributed to these declines. However, pollution also is often cited as a contributing factor [36-39]. Several antiecdysteroids, including testosterone [15], bisphenol A [10], lindane [10], dieldrin [10], and 4-nonylphenol [10], were among the chemicals detected with 3 to 51% frequency among 139 streams sampled during 1999-2000 and analyzed for 95 contaminants [1]. Among these 95 contaminants, most have not been evaluated for antiecdysteroidal activity. Considering that this toxicological property is not rare [10, 13, 15, 40] and that these compounds can synergize, it is possible that some crustacean populations have experienced reduced fecundity from exposure to antiecdysteroids at concentrations of the individual chemicals previously deemed safe.

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Table 1. Influence of one antiecdysteroid on the concentration-response curve of the other. Concentration-response curves were generated for each antiecdysteroid in the presence of subtoxic levels of the other antiecdysteroid. Each treatment consisted of 24 isolated embryos that were exposed to the chemicals for 72 hours. Results are presented as the EC₅₀ values and associated 95% confidence intervals. K values represent the magnitude of shift in toxicant concentration caused by the adjuvant chemical, as measured by the change in EC₅₀ values.

Concentration (μM)		EC ₅₀ (95% CI)		K
Fenarimol	Testosterone	Fenarimol	Testosterone	
0	-	-	13 (11-14)	-
0.5	-	-	12 (11-13)	1.07
1.3	-	-	7.6 (6.3-8.9)	1.72
1.7	-	-	6.3 (5.3-8.1)	2.00
-	0	3.7 (3.4-3.9)	-	-
-	1.5	4.0 (3.7-4.4)	-	0.92
-	3.0	3.4 (3.0-3.8)	-	1.09

Figure legend

Figure 1. Ecdysone levels in daphnid embryos during development. Data are presented as mean and SEM (n = 4-8 groups with each group containing 25-75 individual organisms). Ecdysone levels were normalized to the number of individual organisms (**A**) and to the wet tissue weight of the organisms (**B**). Six embryo developmental stages, as defined previously (Kast-Hutcheson et al., 2001), are divided by dashed lines and indicated numerically.

Figure 2. Ecdysone levels in juvenile daphnids at various times between the first and second molt. Data are presented as mean and SEM (n = 3-6 groups with each group containing 15-25 individual organisms). Ecdysone levels were normalized to the wet tissue weight of the daphnids.

Figure 3. Differential effects of fenarimol and testosterone on embryo development. Total developmental abnormalities (**A**), early developmental abnormalities (**B**), and late developmental abnormalities (**C**) elicited by fenarimol (4.0 μ M) and testosterone (12 μ M). Data are pooled results from two experiments and are presented as the mean $\pm$ SEM (n=6 groups per treatment, 45-75 embryos per group). An asterisk denotes a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's t test).

Figure 4. Developmental abnormalities associated with exposure to fenarimol or testosterone. **A**: normal (control) stage 6 neonatal daphnid. **B**: early developmental abnormality typically

associated with testosterone exposure. **C, D:** late developmental abnormalities consisting of non-distended (**C**) or curved (**D**) shell spine (SS) and underdeveloped second antenna (SA). Late stage developmental abnormalities were associated with fenarimol and testosterone exposure. Daphnids depicted in micrographs **B-D** are the same age as the control organism depicted in 'A'.

Figure 5. Incidence of developmental abnormalities resulting from direct embryonic exposure to concentrations of fenarimol (**A**) and testosterone (**B**). Isolated embryos (24 per treatment group) were exposed to the antiecdysteroid for 72 hours and abnormalities (both early and late) were determined. Concentration-response curves were linearized by log-probit transformation and are accompanied by their 95% confidence intervals. Untreated (control) embryos exhibited no developmental abnormalities.

Figure 6. Comparison of observed embryo toxicity of 30 binary combinations of fenarimol and testosterone with the predicted toxicity based upon the independent joint action (IJA) model (**A**) and the IJA + synergy (IJA+SYN) model (**B**). Linear association lines are accompanied by their 95% confidence intervals. Dashed lines depict the theoretical one-to-one relationship between observed and modeled results.

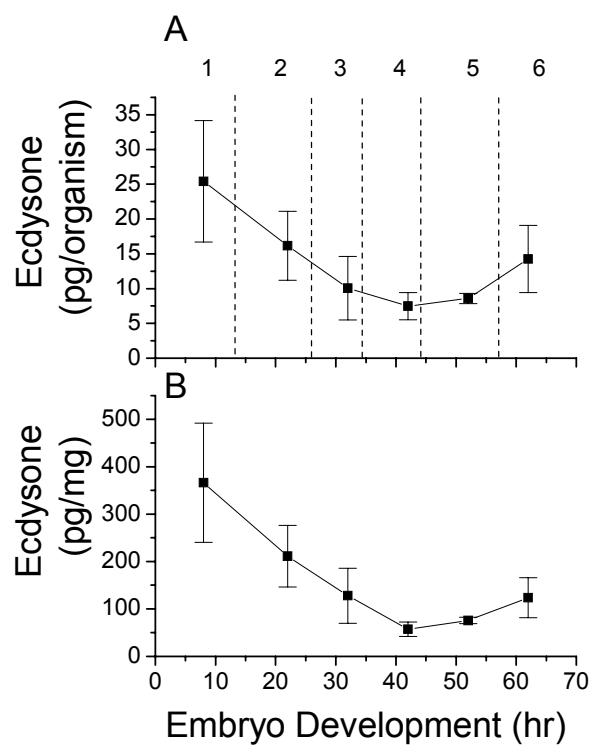


Figure 1

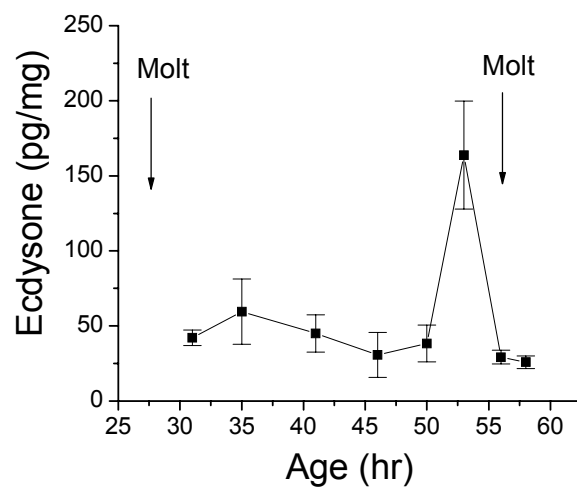


Figure 2

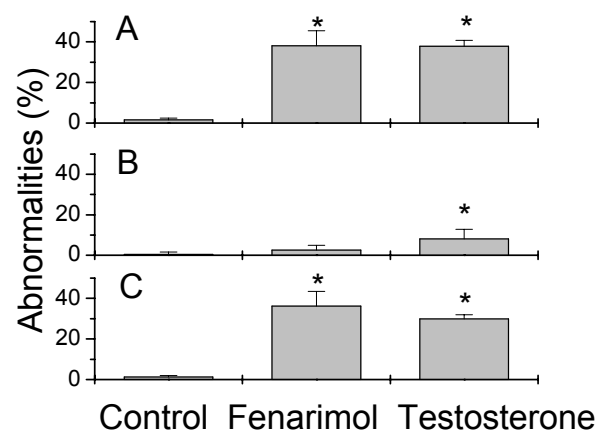


Figure 3

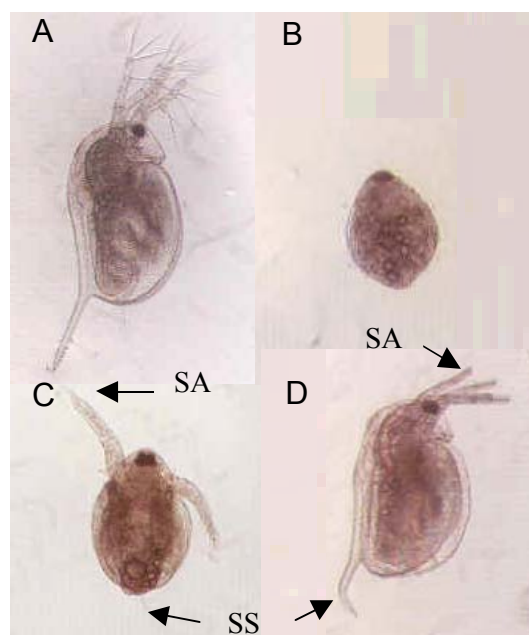


Figure 4

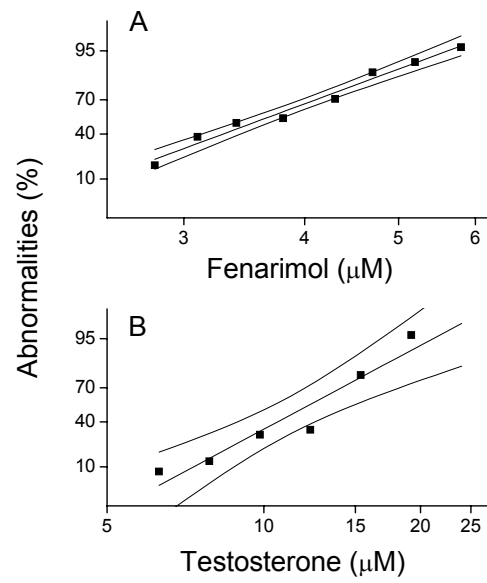


Figure 5

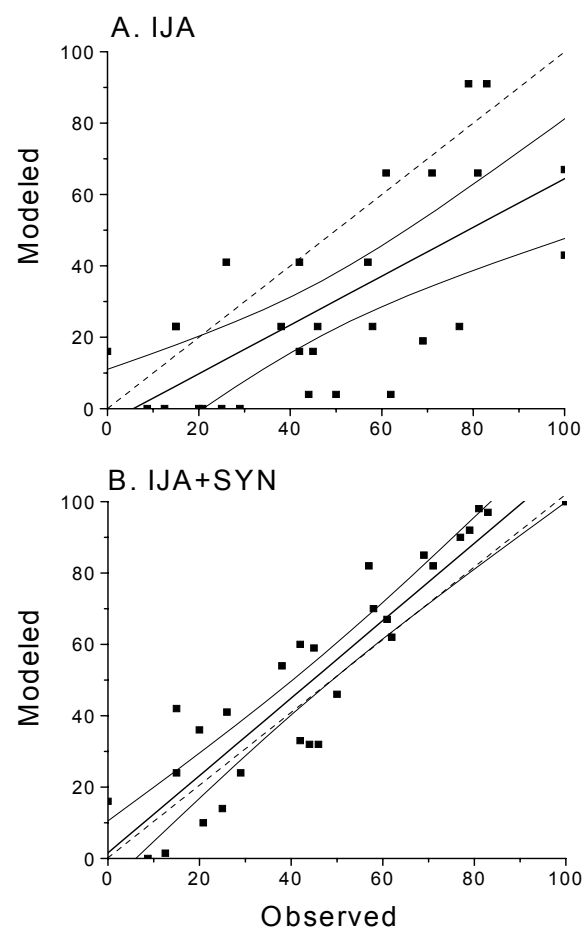


Figure 6

Anti-Ecdysteroidal Activity of Juvenoid Hormones in a Crustacean

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Abstract

Juvenile hormone analogs (JHAs) have been widely used as insect pest control agents, but their effects on non-target crustacean species are largely unknown. We tested the hypothesis that juvenoids can regulate ecdysteroid activity in the crustacean (*Daphnia magna*) in an antagonistic fashion. The crustacean juvenoid hormone methyl farnesoate and the insecticidal JHAs pyriproxyfen and fenoxycarb all disrupted ecdysteroid-regulated aspects of embryo development in daphnids. In vitro exposure of ecdysteroid-responsive cells to 20-hydroxyecdysone reduced cell proliferation and increased mRNA levels of the ecdysone receptor and its partner protein ultraspiracle. Co-treatment of cells with the juvenoid pyriproxyfen attenuated all of the ecdysteroid mediated responses. While juvenoids functioned as anti-ecdysteroids both in vivo and in cultured cells, 20-hydroxyecdysone showed no evidence of acting as an anti-juvenoid. The combined effects of pyriproxyfen and the anti-ecdysteroid fenarimol on embryo development were evaluated in an effort to discern whether these compounds acted in an additive or synergistic manner. The combined action of the juvenoid and anti-ecdysteroid conformed to a model of synergy. These results demonstrated that juvenoids elicit anti-ecdysteroidal activity in a crustacean through a novel mechanism. A model involving receptor partner deprivation is proposed.

Introduction

The endocrinology of insects has been described to a much greater extent than that of any other invertebrate group because of the need to control insect pests, as well as, the industrial application of some insect species (e.g. silkworm) (LeBlanc et al. 1999). In insects, ecdysteroids regulate aspects of development and reproduction, while juvenile hormone (JH) largely modifies the effects of ecdysteroids. For example, in the presence of JH, ecdysteroid pulses cause larval-larval molts; but in the absence of JH, ecdysteroid triggers larval-pupal, then pupal-adult metamorphosis (Riddiford 1994). Juvenile hormone analogs (JHAs) represent a class of insecticides that were designed to specifically disrupt insect development and/or reproduction without impinging on physiological processes in other animals where JH is not present and has no apparent function (Dhadialla et al. 1998). In insects, exposures to JHAs during early embryonic development or final larval stages disrupt development and result in lethal deformities (Riddiford 1994; Staal 1975).

Juvenoid hormones have been shown to block the ability of ecdysteroid to induce the expression of some genes. For example, the juvenoid methoprene has been shown to block ecdysteroid-mediated induction of heat shock proteins (Berger et al. 1992) and microRNAs (Sempere et al. 2003). 20-Hydroxyecdysone can induce (low doses) or suppress (high doses) RNA synthesis in the silk gland of *Galleria mellonella*. However, co-treatment with JHII can block the action of ecdysteroids on RNA synthesis (Kodrik and Sehna 1994). In this context, juvenoids can be characterized as having antiecdysteroid activity.

Recently, we have reported that the ecdysteroids are critical to normal crustacean embryo development and that environmental anti-ecdysteroids can disrupt normal embryo development resulting in reduced fecundity in crustaceans (Mu and LeBlanc 2002a, 2002b). We also have shown that some exogenous chemicals can function as anti-ecdysteroids by lowering endogenous ecdysteroid levels or antagonistic competition with ecdysteroids at the ecdysone receptor (Mu and LeBlanc 2002a, 2002b).

Ecdysteroid action at the molecular level has been well studied in insects. 20-Hydroxyecdysone, the major biologically active ecdysteroid, binds to the ecdysone receptor (EcR) that has heterodimerized with ultraspiracle (USP), another member of the nuclear hormone superfamily. The hormone-receptor complex then binds to the ecdysone response element to activate or inactivate gene transcription (Yao et al. 1993; Cherbas and Cherbas 1996).

JH is generally not considered to be an active hormone in crustaceans. Rather, the precursor to insect JHIII, methyl farnesoate, appears to function as a hormone in these organisms (Laufer et al. 1987). JH and methyl farnesoate regulate many similar processes in insects and crustaceans, respectively (Laufer and Biggers, 2001). Recent research in our laboratory has revealed that methyl farnesoate programs oocytes of daphnids to develop into males (Olmstead and LeBlanc, 2002). Similar activity has been reported for the JHA kinoprene and aphids (Hales and Mittler, 1988).

In the present study, we tested the hypothesis that methyl farnesoate and related JHAs function as anti-ecdysteroids in a crustacean *Daphnia magna* and interfere with ecdysteroid-dependent aspects of embryo development. Experiments also were performed in an attempt to establish the mechanism by which juvenoids interfere with ecdysteroid action. Results indicate that juvenoids interfere with ecdysteroid activity through a novel mechanism that may involve competition for the receptor partner protein USP.

Materials and Methods

Daphnia magna Daphnids were cultured and experimentally maintained in deionized water reconstituted with 192 mg/L $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 192 mg/L NaHCO_3 , 120 mg/L MgSO_4 , 8.0 mg/L KCl, 1.0 $\mu\text{g/L}$ selenium and 1.0 $\mu\text{g/L}$ vitamin B_{12} . Cultures were maintained at a density of 40 brood daphnids/L culture medium. Culture medium was renewed and offspring were discarded three times weekly. Brood daphnids were discarded after 3 weeks in the culture and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1.0 mL (~4 mg dry weight) of Tetrafin[®] fish food suspension (Pet International, Chesterfill, New South Wales, Australia) and 2.0 mL (1.4×10^8 cells) of a suspension of unicellular green algae, *Selenastrum capricornutum*. The algae were cultured in Bold's basal medium. Culture and experimental solutions were maintained at 20°C under a 16-hr photoperiod. These culture conditions maintained the daphnids in the parthenogenic reproductive phase.

Embryotoxicity of juvenoids Experiments were performed to determine whether juvenoids can interfere with ecdysteroid-regulated embryo development of daphnids.

Embryos that were in early stages of development (developmental stage 1 as defined previously (Kast-Hutcheson et al. 2001)), were removed from untreated daphnids as described previously (LeBlanc et al. 2000). Embryos were individually and randomly assigned to wells of 96-well microtiter plates along with 200 μL of medium containing 10 concentrations of the juvenoids pyriproxyfen (Chem Service, West Chester, PA) fenoxycarb

(Chem Service) and methyl farnesoate (Echelon Research Labs), with each concentration being 80% of the next highest concentration. All embryos, including controls, were exposed to the same amount (0.01%) of vehicle (absolute ethanol, AAPER, Shelbyville, KY) used to dissolve the chemicals. Embryos were incubated at 20⁰C under a 16 hr photoperiod and were examined microscopically every 24 hr. Embryos were scored for stage of development and any abnormalities were noted. The percentage of embryos that exhibited developmental abnormalities was determined at the time at which development of the control daphnids was complete. Developmental abnormalities were documented using a digital camera (Pixera Corporation, Los Garos, CA) affixed to the microscope.

Anti-ecdysteroid activity in cultured cells Ecdysone-responsive *Drosophila* Kc cells (kindly provided by Drs. Lucy and Peter Cherbas, Indiana University, Bloomington) were cultured in Schneider's medium (Sigma, St. Louis, MO) with 5% fetal calf serum (Sigma, St. Louis, MO) as previously described (Cherbas and Cherbas, 1998). In the absence of ecdysteroids, these cells are spherical shaped and readily proliferate. In the presence of ecdysteroids, these cells cease to proliferate and become spindle-shaped, elongate, and emit long pseudopodia (Cherbas et al. 1989). Cells were plated in 96-well dishes at a density of $1-2 \times 10^5$ cells/mL. Cells were cultured in 1.0 μ M 20-hydroxyecdysone alone or 20-hydroxyecdysone with increasing concentrations of the juvenoid pyriproxyfen. All exposure solutions, including controls, contained 0.5% ethanol. Cells were harvested after 3 days and counted using a hemocytometer.

Ecdysteroid receptor (EcR) and ultraspiracle (USP) mRNA quantification Kc Cells were cultured in 1.0 μ M 20-hydroxyecdysone alone, 20-hydroxyecdysone combined with 10 nM pyriproxyfen or 10 nM pyriproxyfen alone. All exposure solutions, including controls, contained 0.5% ethanol. Cells were harvested after 3 days. Total RNA was isolated using Promega SV Total RNA Isolation System (Madison, WI). cDNAs were made from the respective RNAs with oligo (dT) primer using Promega Improm-IITM Reverse Transcription System (Madison, WI). Quantitative Real-time PCR was performed on an ABI prism 7000 sequence detection system (Applied Biosystems) using default parameters, specifically, the reaction mixtures were first kept at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. SYBR Green PCR Master Mix (Applied Biosystems) was used in the PCR reactions described above. After the PCR reaction, samples were run for the dissociation protocol and a single melting peak was obtained for all samples showing no amplification of non-target DNA. PCR primers were designed using Primer Express software (Applied Biosystems) as following: EcR forward primer 5'-CAAAATGACCACTTCGCCG-3', reverse primer 5'-GGCCAAGCTGCCATTGC-3'; USP forward primer 5'-TGGCAAGCACTACGGCGT-3', reverse primer 5'-TTAAAGAAGCCCTTGCAGCC-3'. The threshold cycle (Ct) was used for assessing the relative levels of EcR and USP to the transcript of the control actin gene. The actin PCR primers were forward primer 5'-GGTCGGTATGGGCCAGAAG -3', reverse primer 5'- TTGGACTGGGCCTCATCAC -3'.

Male-sex determination during oocyte exposure The juvenoids methyl farnesoate, pyriproxyfen and methoprene were shown to program maturing oocytes in the ovary to

develop into males (Olmstead and LeBlanc 2003). A similar experimental design was used to determine if ecdysteroids can disrupt this juvenoids-regulated activity. Maternal daphnids were exposed to concentrations of 20-hydroxyecdysone or juvenoid alone or 20-hydroxyecdysone/juvenoid combinations during oocyte maturation, and sex of the resulting progeny was determined. Should 20-hydroxyecdysone disrupt the activity of juvenoids, then 20-hydroxyecdysone should interfere with the ability of the juvenoids to program oocyte to develop into males. Adult female daphnids carrying embryos in their brood chambers were selected from the cultures and placed individually in 50-ml beakers containing 40 ml media containing the appropriate concentrations of the test chemicals. The daphnids were maintained in this solution for 24 hr, which encompassed the sex-determination period of ovarian oocyte maturation. Daphnids then were transferred to clean medium and maintained until the brood of offspring exposed to test chemical(s) in the ovary was released. Food was provided to each beaker twice daily as 0.05 mL (~0.2 mg dry weight) of Tetrafin[®] fish food suspension and 0.1 mL (7×10^6 cells) of a suspension of unicellular green algae, *Selenastrum capricornutum*. Daphnids typically produce only female offspring under these culture conditions. Sex of individual offspring was determined microscopically with males being discerned from females by the longer primary antennae (Olmstead and LeBlanc 2003).

Combined effect of pyriproxyfen with a known antiectdysteroid Potential interactive effects of the pyriproxyfen and fenarimol, a known anti-ecdysteroid, on ecdysteroid-dependent embryo development were evaluated by isobolic analyses (Christensen and Chen 1991). Experiments were performed using the ex vivo embryo exposure methods described

previously under “embryotoxicity of juvenoids”. Embryos were exposed to serial concentrations of fenarimol or pyriproxyfen alone, or the concentrations of fenarimol in the presence of 4 concentrations (0.04, 0.13, 0.40, 2.46 nM) of pyriproxyfen. EC₅₀ values of fenarimol or pyriproxyfen alone, or fenarimol combined with pyriproxyfen were calculated by probit analysis (PROC PROBIT, SAS 8.0 software, SAS institute, Cary, NC).

Statistics Statistical significance was evaluated by Analysis of Variance and Dunnett’s t-test when comparing multiple treatments to the control using JMP software (SAS Institute, Cary, NC). The homogeneity of variances of the data was established by Bartlett’s test. Concentration–response curves were generated by sigmoidal fit using Origin software (Microcal Software Inc., Northampton, MA).

Results

Embryotoxicity of juvenoids

Three juvenoids were evaluated for their ability to interfere with ecdysteroid-regulated embryo development in daphnids. All three juvenoids caused developmental abnormalities consistent with anti-ecdysteroidal activity (Fig. 1). Specifically, these compounds caused curvature of the shell spine (Fig. 2). Pyriproxyfen and fenoxycarb were equipotent at disrupting embryo development. Methyl farnesoate was approximately 2 orders of magnitude less potent than pyriproxyfen and fenoxycarb. These results demonstrate that juvenoids can interfere with ecdysteroid-regulated embryo development.

Anti-ecdysteroid activity of juvenoids

We are not aware of any ecdysone-responsive crustacean cell lines. Therefore, experiments were performed with ecdysone-responsive *Drosophila* Kc cells to determine whether juvenoids disrupt the ecdysteroid signal transduction pathway. Such disruption could explain the adverse effects of juvenoids on crustacean embryo development. Proliferation of Kc cells was significantly reduced by 1.0 μ M 20-hydroxyecdysone (Fig. 3). Exposure to increasing concentrations of pyriproxyfen progressively abrogated the suppressive effect of 20-hydroxyecdysone on cell proliferation (Fig. 3). However, cell proliferation was not completely restored at the pyriproxyfen concentrations evaluated. These observations indicated that juvenoids can interfere with the ability of ecdysteroids to regulate cellular processes.

The ecdysone receptor (EcR) must dimerize with ultraspiracle (USP) to form a functional transcription factor. Quantitative Real-time PCR revealed that EcR and USP both were constitutively expressed in the Kc cells (Fig. 4). Exposure to 20-hydroxyecdysone significantly elevated levels of both EcR and USP (Fig. 4). Pyriproxyfen ameliorated this effect of 20-hydroxyecdysone. Pyriproxyfen alone had no discernable effect on the expression of either EcR or USP. These results further demonstrated that juvenoids interfered with the molecular actions of ecdysteroids, and identified a potential mechanism (reduced ecdysteroid receptor gene expression) that could contribute to this effect.

Effect of ecdysteroid on juvenoids-driven male-sex determination during oocyte maturation

Having demonstrated that a juvenoid can interfere with ecdysteroid regulated activity in both intact daphnids and cultured Kc cells, next we evaluated the possibility that ecdysteroids can disrupt juvenoids-regulated activity using male-sex determination during oocyte exposure as the juvenoid-regulated endpoint (Olmstead and LeBlanc, 2003). Such reciprocal regulation would imply a bidirectional cross talk between these two signaling pathways. 20-Hydroxyecdysone elicited little intrinsic juvenoid activity as indicated by its inability to stimulate a significant level of male offspring production upon exposure of maternal daphnids (Fig. 5A). 20-Hydroxyecdysone did exhibit slight juvenoid activity in the presence of a low concentration of the juvenoid methyl farnesoate (Fig. 5B). This effect is

characteristic of a weak juvenoid. 20-Hydroxyecdysone did not elicit anti-juvenoid activity as indicated by its inability to inhibit the pyriproxyfen-mediated stimulation of male-offspring production (Fig. 5C). Thus, juvenoids do not elicit anti-ecdysteroid through some bidirectional mechanism of action.

Combined effect of pyriproxyfen with a known anti-ecdysteroid fenarimol

The combined anti-ecdysteroidal effects of the ecdysteroid-lowering compound fenarimol and pyriproxyfen were evaluated to determine whether the combined effects of these compounds better conformed to a model of additivity or synergy. An additive response would imply that pyriproxyfen elicited anti-ecdysteroidal activity by suppressing some discrete component of the ecdysteroid signaling pathway (i.e., lowering ecdysteroid or ecdysteroid receptor levels). However, a synergistic response would indicate a mechanism of anti-ecdysteroidal activity elicited by the juvenoid that enhances or is enhanced by the action of fenarimol. Isobolic analysis revealed that binary combinations of fenarimol and pyriproxyfen did not conform to a model of additive but rather was strongly indicative of synergy (Fig. 6). Thus, the mechanism of anti-ecdysteroidal activity of the juvenoid involves more than simple suppressive action at some discrete component of the ecdysteroidal signaling pathway.

Discussion

The hypothesis was tested that juvenile hormones elicit anti-ecdysteroidal activity in crustaceans. This hypothesis was tested by evaluating the effect of several juvenoids on ecdysteroid-dependent aspects of embryo development of *Daphnia magna*. Anti-ecdysteroidal activity of juvenoids was further evaluated using an ecdysone-responsive insect cell. A comparable crustacean cell line was not available for these experiments. Ecdysteroid-dependent embryo development, arrest of cell proliferation, and gene induction all were blocked by co-administration of juvenoid. Based upon these observations, the proposed hypothesis was accepted.

The order of potency with which the various juvenoids evaluated elicited anti-ecdysteroidal activity was the same as previously determined when measuring the action of these hormones as a male sex determinant in daphnids (Olmstead and LeBlanc 2003 and unpublished data). However, all juvenoids were approximately ten-times less potent at eliciting anti-ecdysteroidal activity as compared to sex determination. These observations imply that the anti-ecdysteroidal activity and male sex determining activity of juvenoids involve somewhat different molecular interactions.

HR38 is a member of the nuclear receptor superfamily that has been identified in *Drosophila* (DHR38), mosquito (AHR38), and silkworm (BHR38) (Sutherland et al. 1995; Zhu et al. 2003; Matsuoka and Fujiwara 2000). The receptor has been shown to negatively regulate the

action of 20-hydroxyecdysone by competing with EcR for dimerization to USP (Sutherland et al. 1995; Zhu et al. 2003). Involvement of HR38, or a related receptor protein, in the anti-ecdysteroidal activity of juvenoids (Fig. 7) would account of many of the observations made in this study. For example, methyl farnesoate and JHAs may induce expression of HR38 which then dimerizes with USP and suppresses EcR transcriptional activity (Fig. 7). Under this scenario, relative potency of the juvenoids regarding both anti-ecdysteroidal activity and male sex determination would be dictated by binding affinity to the methyl farnesoate receptor (MfR). However, anti-ecdysteroidal activity, but not male-sex determination, also would dependent upon the extent to which USP is successfully withheld from dimerization to EcR. This secondary requirement could explain the need for higher concentrations of juvenoids required for this activity.

The MfR also may directly heterodimerize with USP upon binding its juvenoid ligand (Fig. 7). The implications of such interaction with respect to anti-ecdysteroidal consequences, potency differences among juvenoids, and the need for higher levels of juvenoid to elicit anti-ecdysteroidal activity relative to induction of male-sex production would all be similar to that described with HR38 involvement. Finally, we cannot exclude the possibility that MfR and USP are the same protein and binding of juvenoid to MfR/USP prevents its dimerization to EcR. Again, the consequences would be similar to that described under the previous scenarios. Precedence exists for USP having the capability to bind juvenoids (Jones and sharp 1997).

Results of this study demonstrated that juvenoids can function as anti-ecdysteroids in crustaceans. However, we found no evidence for ecdysteroids having the ability to function as anti-juvenoids. If indeed the mechanism by which juvenoids inhibit ecdysteroid activity is through partner receptor (USP) deprivation, then results also suggest that ecdysteroids are not similarly capable of preventing activation of the juvenoid transcription factor. This putative unidirectional crosstalk between two signaling pathways may be due to greater susceptibility of the EcR receptor dimer to dissociation and may be important in the overall modulation of ecdysteroid activity by juvenoids.

Experiments that were performed to measure effects of ecdysteroids on a juvenoid-regulated process revealed weak juvenoid activity associated with 20-hydroxyecdysone. Treatment of Kc cells with 20-hydroxyecdysone also was shown to elevate USP expression. Should USP be involved in mediating the activity of the juvenoids (Fig. 7) then the induction of USP by 20-hydroxyecdysone could be responsible for the weak juvenoid activity measured. Indeed, the requirement for exogenous juvenoid along with 20-hydroxyecdysone would support a model whereby ecdysteroid induces receptor that must bind added juvenoid to signal the production of male offspring.

The involvement of a multi-component mechanism (regulation of multiple genes, receptor partner deprivation) in the anti-ecdysteroidal activity of juvenoids is further implicated from the results of the combination experiments with pyriproxyfen and the ecdysteroid-lowering anti-ecdysteroid fenarimol. If the juvenoid had elicited anti-ecdysteroidal activity exclusively

by lowering ecdysteroid levels or lowering EcR levels then the combined anti-ecdysteroidal activity of the two compounds would have conformed to an additivity model. In contrast, if the juvenoid interacted with the ecdysteroid receptor in a manner that reduced receptor interaction with the remaining ecdysteroid following fenarimol depletion of the ecdysteroid pool, then observed effects would conform to a model of synergy. A strong synergistic interaction was observed between the two chemicals. As a mechanism of synergy, we propose that juvenoids promote dissociation of EcR-USP that is not yet associated with ecdysteroid ligand. We propose that ecdysteroid binding to the EcR-USP heterodimer strengthens the association between the two proteins and reduces the ability of juvenoid to cause dissociation. Reduced ecdysteroid levels, due to fenarimol treatment, increases the availability of USP for withdraw from EcR association and thus increases the potency with which juvenoids can withhold USP from the ecdysteroid receptor complex. This EcR-conferred increased potency of juvenoid could account for the observed synergistic interaction between the two anti-ecdysteroids. Reduced ecdysteroid levels resulting from fenarimol treatment also would attenuate the ecdysteroid-mediated increase in EcR and USP receptor levels as measured in the present study. Reduced receptor levels would further enhance anti-ecdysteroid activity by decreasing available receptor to participate in transcriptional activation of ecdysteroid-responsive genes and may increase the proportion of USP that is withheld from EcR.

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Figure legends

Figure 1. Incidence of developmental abnormalities resulting from in vitro embryo exposure to concentrations of juvenoids: **(A)** methyl farnesoate, **(B)** fenoxycarb, and **(C)** pyriproxyfen. Each data point represents the mean incidence of developmental abnormalities among 24 embryos. The incidence of developmental abnormalities among control embryos is depicted as an open circle.

Figure 2. Developmental abnormality associated with exposure to juvenoids. **(A)** Normal (control) stage 6 neonatal daphnid. **(B)** Developmental abnormality typically associated with juvenoid exposure. Arrow indicates the curved shell spine.

Figure 3. Antagonistic activity of pyriproxyfen (PP) on the anti-proliferative action of 20-hydroxyecdysone (20E) on Kc cells. Data are presented as the mean $\pm$ SEM (n=3 groups per treatment). An asterisk denotes a significant difference from the 20E treatment alone at $p \leq 0.05$ (ANOVA, Dunnett's t test).

Figure 4. Influence of pyriproxyfen (PP) on the 20-hydroxyecdysone (20E)-induced gene expression of **(A)** ecdysone receptor (EcR) and **(B)** ultraspiracle (USP). In each treatment, the mRNA level of target gene was normalized to the transcript of housekeeping gene actin. Data are presented as mean and SEM (n=3). An asterisk denotes a significant difference from the respective control at $p \leq 0.05$ (ANOVA, Dunnett's t test).

Figure 5. Effect of 20-hydroxyecdysone (20E) on juvenoids-driven male-sex determination during oocyte exposure. **(A)** The intrinsic juvenoid activity of 20E. Data are presented as the mean $\pm$ SEM (n=4 experiments, each individual treatment consisting of 8-13 broods). **(B)** The juvenoid activity of 20E in the presence of a low level of methyl farnesoate (MF). Data are presented as the mean $\pm$ range of variation (n=2 experiments, each individual treatment consisting of 8-13 broods). **(C)** The anti-juvenoid activity of 20E in the presence of high level of pyriproxyfen (PP) (n=1 experiment, each treatment consisting of 10 broods).

Figure 6. The EC50 isobole for pyriproxyfen and fenarimol mixtures resulting from in vitro embryo exposure. Dotted line depicts the predicted location of the data should the compounds conform to a model of simple additivity. An isobole that falls below the additivity line is indicative of synergy; while, an isobole that locates above the additivity line is indicative of antagonism. The error bars represent 95% confidence interval for the derived EC50 values.

Figure 7. Some proposed mechanisms by which juvenoids (ie., methyl farnesoate (MF)) could stimulate receptor dimerization between USP and HR38 or USP and MfR resulting in receptor partner (USP) deprivation to the ecdysteroid receptor (EcR) and loss of EcR signaling activity. E = ecdysteroid, HR38 = hormone receptor 38.

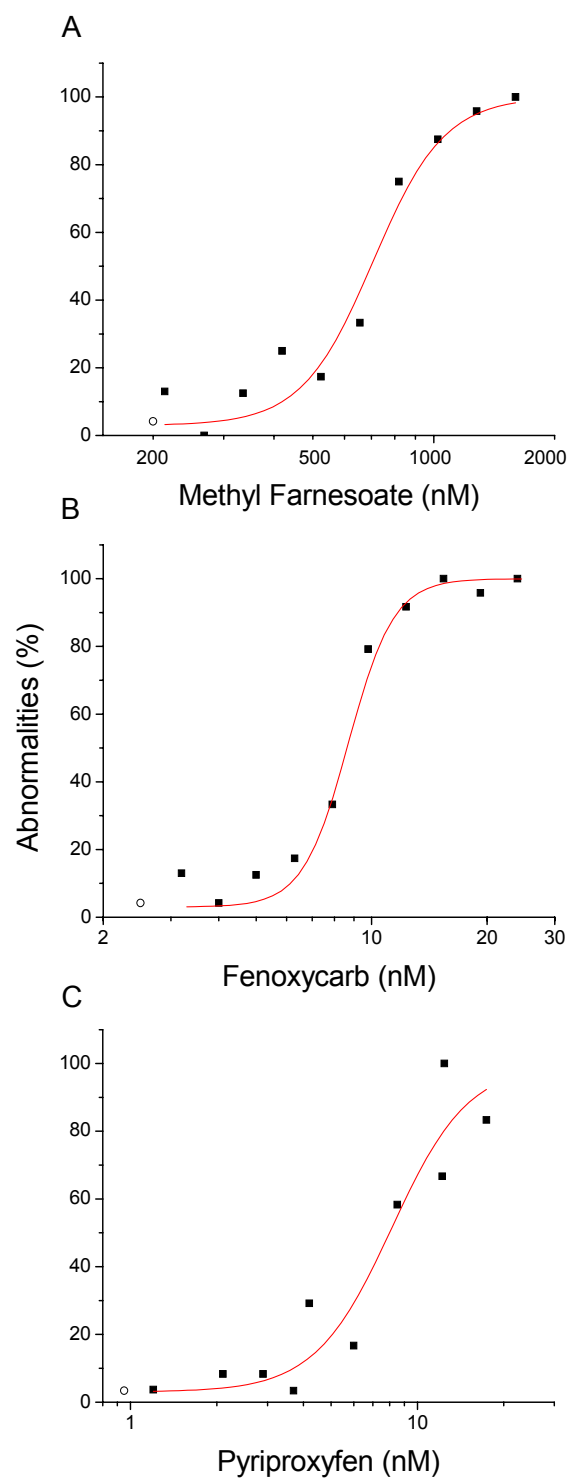


Figure 1



Figure 2

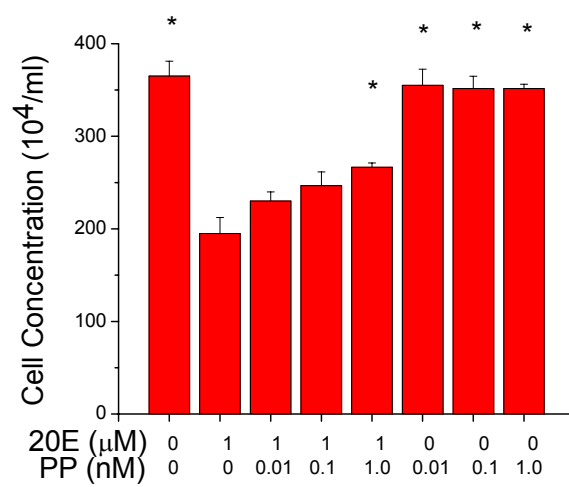


Figure 3

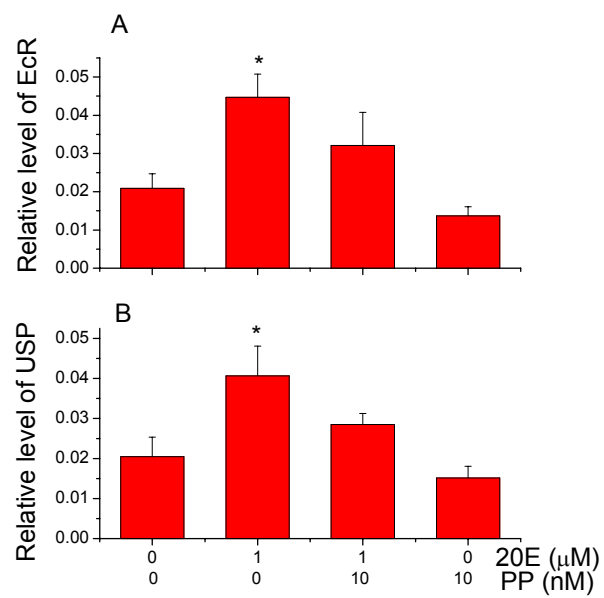


Figure 4

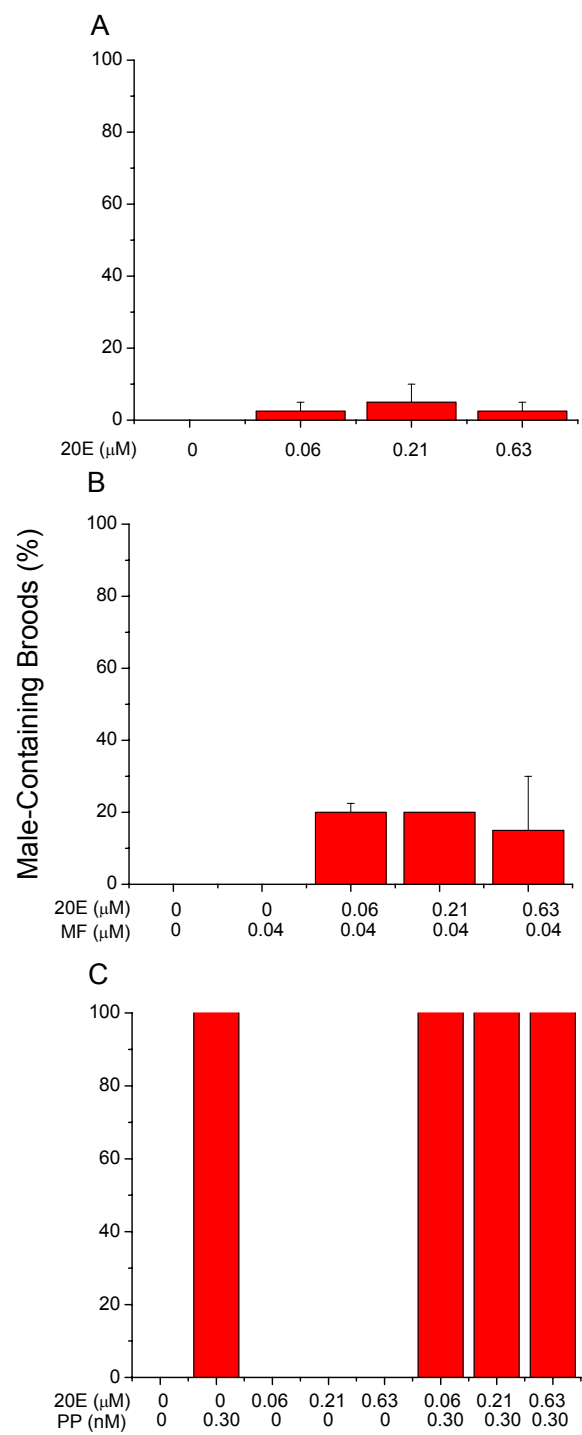


Figure 5

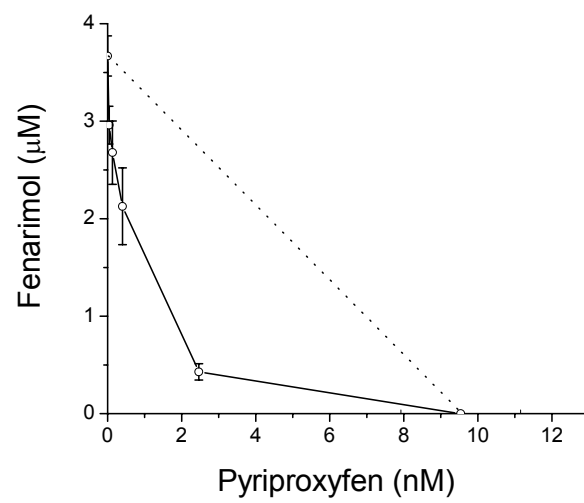


Figure 6

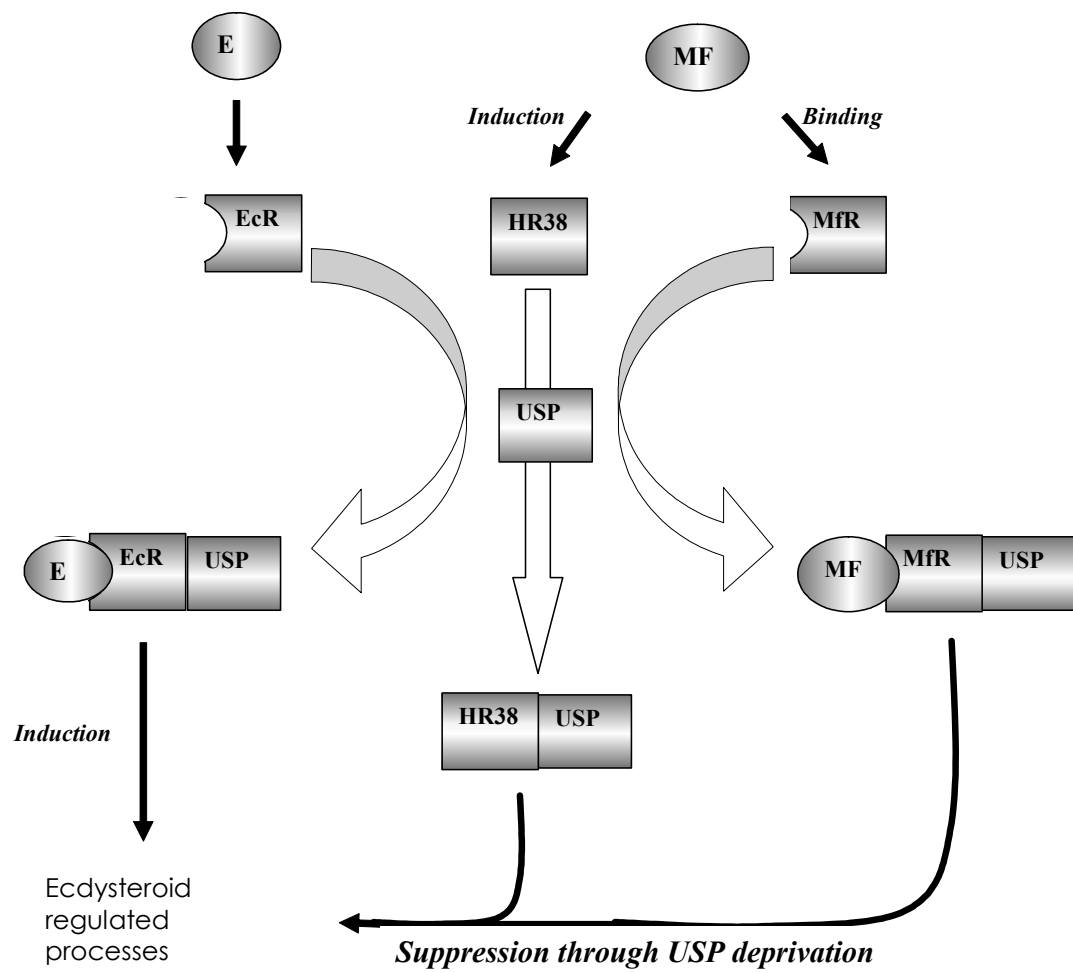


Figure 7

Anti-Ecdysteroidal Activity of Bisphenol A to a Crustacean

Daphnia magna

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Abstract

Bisphenol A is a key industrial chemical used in the manufacture of polycarbonate plastics, epoxy resins and other products. Several recent reports ascribe toxicological properties to this compound that have been attributed the disruption of endocrine-related processes. In the present study, the toxicity of bisphenol A was definitively characterized in the water flea (*Daphnia magna*) in an effort to discern whether this compound may elicit endocrine-related toxicity in an invertebrate species at environmentally-relevant concentrations. The ability of bisphenol A to interfere with two ecdysteroid-dependent physiological processes – molting and embryo development - was evaluated. Bisphenol A elicited anti-ecdysteroidal activity as indicated by its prolongation of the intermolt period and interference with embryo development. This apparent anti-ecdysteroidal activity was neither due to reduced availability of endogenous ecdysteroid nor due to ecdysteroid receptor antagonism. Juvenoid hormones are known to function as anti-ecdysteroids, therefore, the ability of bisphenol A to elicit juvenoid activity was evaluated. Bisphenol A elicited weak juvenoid activity, as evidenced by its stimulation of male sex determination of offspring – a juvenoid regulated process – when co-administered with the juvenoid hormone methyl farnesoate. A definitive assessment of the effects of bisphenol A on the reproductive capacity of daphnids revealed a concentration-response relationship that extended at least one order-of-magnitude below exposure levels that were overtly toxic to the maternal organisms. These results demonstrate that bisphenol A is chronically toxic to daphnids, probably through its anti-ecdysteroidal/juvenoid activity. However, effects are elicited at levels that are not likely to pose environmental concern.

INTRODUCTION

The characterization of endocrine toxicity has focused primarily upon the effects of environmental chemicals on androgen, estrogen, and thyroid hormone dependent-processes. This emphasis is predicated by the fact that these three hormone systems are intricately involved in regulating development, growth, and reproduction in vertebrates and thus provide a good first attempt at detecting and regulating endocrine-disrupting toxicity (EPA 2001). However, the use of these signaling molecules in invertebrates is equivocal while other signaling molecules are clearly operative in invertebrates but are not physiologically relevant in vertebrates (LeBlanc, Cambell et al. 1999).

Among these invertebrate hormones are ecdysteroids. Ecdysteroids represent a class of steroid hormones in arthropods (insects, crustaceans, and some minor phyla) that regulate many aspects of development, growth, and reproduction that are associated with androgens, estrogens, and thyroid hormone in vertebrates. For example, embryo development in crustaceans is regulated in part by ecdysteroids (Mu and LeBlanc 2002a). Growth in arthropods requires periodic loss of the exoskeleton and replacement with a larger cuticle. This molting process is regulated by ecdysteroids (Horn, Middleton et al. 1966) and thus these hormones are permissive of growth. Ecdysteroids also have been shown to regulate aspects of vitellogenesis and spermatogenesis therefore also function as reproductive hormones (Gohar and Souty 1984; Happ 1992; Adams 1999).

Ecdysteroids function through receptor activation in a manner orthologous to the vertebrate thyroid hormone receptor. The ecdysone receptor (EcR) functional activation is mediated in part through its dimerization with a partner receptor ultraspiracle (USP) and binding of the ecdysone ligand (typically 20-hydroxyecdysone) (Yao, Segraves et al. 1992; Yao, Forman et al. 1993). USP is homologous to the vertebrate RXR receptor (Khoury Christianson, King et al. 1992; Yao, Forman et al. 1993). This ligand-dimer complex then regulates activity of ecdysone-responsive genes by interacting with cis-acting elements of the genes.

Environmental chemicals that have the potential to bind EcR as agonists or antagonists have the potential to elicit toxicity unique to arthropods. Indeed, some insecticides, such as tebufenozide, elicit highly specific effects on target insects based upon their ability to bind the EcR as agonists (Dhadialla, Carlson et al. 1998). We recently reported that testosterone functions as an EcR antagonist and accordingly interferes with the ecdysteroid-regulated processes of molting and embryo development in the crustacean *Daphnia magna* (Mu and LeBlanc 2002b). The industrial chemical and environmental contaminant bisphenol A was reported to function as weak ecdysteroid antagonists in a cell-based assay (Dinan, Bourne et al. 2001a). However, we are aware of no reports of potential environmental contaminants that elicit toxicity to an arthropod species by functioning as an antiecdysteroid.

Chemicals that lower endogenous ecdysone levels or function as ecdysone antagonist in daphnids cause abnormal embryo development resulting in reduced production of viable offspring (Mu and LeBlanc 2002a; Mu and LeBlanc 2002b). The mechanistic specificity of such toxicity may render arthropods particularly sensitive to the toxicity of such materials. We hypothesized that bisphenol A would interfere with ecdysteroid-dependent embryo development of daphnids resulting in reduced fecundity.

Bisphenol A is an industrial chemical used in the manufacture of polycarbonate plastics, epoxy resins and other products. This compound has been shown interact with the estrogen receptor as an agonist and elicit estrogen-like activity in vivo (Krishnan, Stathis et al. 1993; Colerangle and Roy 1997; Markey, Luque et al. 2001; Ramos, Varayoud et al. 2001). This compound also has been shown to interact with the mammalian androgen receptor in an antagonistic manner (Lee, Chattopadhyay et al. 2003). The material is ubiquitous in the aquatic environment with concentrations typically measured in the parts per billion range (discussed in Oehlmann, Schulte-Oehlmann et al. 2000).

MATERIALS AND METHODS

Daphnids

Daphnids were cultured and experimentally maintained in deionized water reconstituted with 192 mg/L $\text{CaSO}_4 \cdot \text{H}_2\text{O}$, 192 mg/L NaHCO_3 , 120 mg/L MgSO_4 , 8.0 mg/L KCl, 1.0 $\mu\text{g/L}$ selenium and 1.0 $\mu\text{g/L}$ vitamin B_{12} . Cultures were maintained at a density of 40 brood

daphnids/L culture medium. Culture medium was renewed and offspring were discarded three times weekly. Brood daphnids were discarded after 3 weeks in the culture and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1.0 mL (~4 mg dry weight) of Tetrafin[®] fish food suspension (Pet International, Chesterfill, New South Wales, Australia) and 2.0 mL (1.4×10^8 cells) of a suspension of unicellular green algae, *Selenastrum capricornutum*. The algae were cultured in Bold's basal medium. Culture and experimental solutions were maintained at 20°C under a 16 hr photoperiod. These culture conditions maintained the daphnids in the parthenogenic reproductive phase.

Ecdysteroid dependent endpoints during continuous exposures

Experiments were performed to determine whether bisphenol A elicited anti-ecdysteroidal activity in vivo as in dictated by altering the ecdysteroid-dependent endpoints: intermolt duration and embryo development.

Intermolt duration Individual female daphnids (<1 hr old) were exposed to each of 20 concentrations of the chemicals with each concentration being 90% of the next highest concentration. Exposure concentrations were selected in which the highest concentration approximated the maximum tolerated concentration as measured during preliminary experiments. Exposures were conducted in 50-mL beakers containing 40 mL of medium. All treatments, including controls, contained the same amount (0.01%) of vehicle (absolute ethanol, AAPER, Shelbyville, KY) used to dissolve the chemicals. Controls for each experiment consisted of 10 beakers each containing a single daphnid. Daphnids were

provided food (0.05 mL (~0.2 mg dry weight) of Tetrafin[®] fish food suspension and 0.1 mL (7×10^6 cells) of a suspension of unicellular green algae, *S. capricornutum*) and test solutions were renewed every twenty four hours. Vessels were examined hourly for the presence of a molted exoskeleton and time of molting was recorded to the nearest hour for each daphnid. The duration of the first intermolt period (time between birth and molting to the nearest hour) for each daphnid was plotted against exposure concentration and judged to best conform to either a single segmented line or a two segmented line. Three possible relationships were considered. 1) A single segmented line having no slope would be indicative of the chemical having no effect on intermolt duration. 2) A single segmented line having a significant slope and a significant positive relationship between exposure concentration and intermolt duration would indicate an antiecdysteroid-like effect with no threshold concentration. 3) A two segment line consisting of relationship 1 followed by relationship 2 would be indicative of antiecdysteroidal effects with a threshold concentration.

The ability of 20-hydroxyecdysone to protect daphnids against the effects of bisphenol A on molting was assessed using these same basic procedures except that the effect of each treatment was evaluated using the mean $\pm$ standard deviation response of eight neonatal daphnids. Both bisphenol A and 20-hydroxyecdysone were delivered to the media in ethanol at a final total ethanol concentration of 0.01%. Control solutions also contained this amount of ethanol. Significant differences among treatments were evaluated using analysis of variance (ANOVA) and Dunnett's t test (Gad 1998) at $P \leq 0.05$.

Embryo development Neonatal daphnids were exposed to concentrations of bisphenol A using the same basic experimental design as described for ‘intermolt duration’ experiments with the following modifications. Daphnids were provided food daily as described above and exposure solutions were renewed three times weekly. Daphnids began releasing broods of parthenogenically-reproduced offspring on approximately day 7, each beaker was examined daily thereafter for released offspring. Offspring were counted, removed from the beakers, and examined microscopically to identify any developmental abnormalities. Experiments were terminated after 21 days of exposure.

Isolated embryo exposures

Embryos of the same age (as indicated in the specific experiments), were removed from untreated maternal daphnids as described previously (LeBlanc, Mu et al. 2000). Embryos were individually and randomly assigned to wells of 96-well microtiter plates along with 200 µL of medium containing various concentrations of bisphenol A. All embryos, including controls, were exposed to the same concentration of ethanol vehicle (0.01%). Embryos were incubated at 20⁰C under a 16 hr photoperiod and were examined microscopically every 24 hr. Embryos were scored for stage of development and any abnormalities were noted. The percentage of embryos that exhibited developmental abnormalities was determined at the time at which development of the control daphnids was complete. The number of embryos used per treatment level is indicated in the Results section for individual experiments.

The ability of 20-hydroxyecdysone to protect embryos against the developmental toxicity of bisphenol A was assessed with the same basic experimental design as used to assess the developmental toxicity of bisphenol A, but at a single concentration of bisphenol A with concurrent exposure to increasing concentrations of 20-hydroxyecdysone.

Male sex determination

Male sex determination is a juvenoid-regulated process in daphnids (Olmstead and LeBlanc 2002). Juvenoid activity of bisphenol A was measured by its ability to stimulate the production of male offspring by treated maternal organisms. Maternal daphnids were exposed to concentrations of bisphenol alone or in the presence of a juvenoid hormone during ovarian oocyte maturation, and sex of the resulting progeny was determined. Adult female daphnids carrying embryos in their brood chambers were selected from the cultures and placed individually in 50-ml beakers containing 40 ml media containing the appropriate concentrations of the test chemicals. The daphnids were maintained in this solution for 24 hr, which encompassed the sex-determination period of ovarian oocyte maturation. Daphnids then were transferred to chemical-free medium and maintained until the brood of offspring exposed to test chemical(s) in the ovary was released. Food was provided to each beaker twice daily as described above. Daphnids typically produce only female offspring under these culture conditions. Sex of individual offspring was determined microscopically with males being discerned from females by the longer primary antennae (Olmstead and LeBlanc 2001).

Maternal fecundity

Maternal fecundity was assessed using the same procedures as described under ‘embryo development’. Concentration-dependent effects of bisphenol A on total offspring produced per female over the 21-day experiment were evaluated by linear regression as described for the ‘intermolt duration’ experiments.

Acute toxicity assessments

The acute toxicity of bisphenol A to daphnids was evaluated using standard procedures (Stephan 1975). Daphnids (<24 hrs old) were exposed to concentrations of the chemical for 48 hours and adverse effects (immobilization, lethargy, death) was determined. Exposure concentrations were selected that were expected to bracket the EC₅₀ value, based upon preliminary experiments. For each treatment level, two 50 mL beakers containing 40 mL of solution and 10 daphnids were used. The chemicals were delivered to the media (same as culture media) at the desired concentration dissolved in absolute ethanol. All solutions and controls for a specific experiment contained the same concentration of ethanol ($\leq 0.01\%$). Vessels were maintained in a temperature and light controlled incubator as described for cultures. After 48 hours, daphnids were examined for effects and results used to calculate EC₅₀ values and accompanying 95% confidence intervals using Origin software (MicroCal Software Inc, Northhamton, MA).

RESULTS

Anti-ecdysteroidal activity of bisphenol A during continuous exposure

Experiments initially were performed to determine whether daphnids that were continuously exposed to concentrations of bisphenol A exhibited symptoms of toxicity that were consistent with antiecdysteroidal activity. Specifically, effects of bisphenol A on the time duration between exuviations and on development of offspring were evaluated. The period of time between the first and second exuviations progressively increased with increasing bisphenol A concentrations greater than approximately 5 mg/L (Fig. 1). Further, exposure to 10 mg/L bisphenol A caused an increased incidence of developmental abnormalities among offspring (Fig. 2). In combination, these observations suggest that, at high exposure levels, bisphenol A elicits toxicity indicative of anti-ecdysteroidal activity.

Protection by 20-hydroxyecdysone

Among the mechanisms by which a chemical may elicit anti-ecdysteroidal activity are the lowering of endogenous ecdysteroid titers and competitive antagonism at the ecdysone receptor. In either case, the antiecdysteroidal effect of the chemical can be ameliorated by the provision of exogenous 20-hydroxyecdysone. Experiments were therefore performed to determine whether daphnids could be rescued from the adverse effects of bisphenol A by co-exposure to 20-hydroxyecdysone. Exposure of daphnids to 9.0 mg/L bisphenol A through the first intermolt period significantly increased the duration of this period (Fig. 3).

However, co-exposure to concentrations of 20-hydroxyecdysone had no effect on this action of bisphenol A (Fig. 3). Similarly, exposure of isolated embryos to bisphenol A increased the incidence of developmental abnormalities, but co-exposure to 20-hydroxyecdysone had no protective effect towards the embryos (Fig. 4). Rather, 20-hydroxyecdysone enhanced the developmental toxicity of bisphenol A (Fig. 4). During the performance of these experiments, it also became evident that susceptibility of embryos to the developmental toxicity of bisphenol A increased with decreasing age of the embryos (Fig. 4 panels A versus B, Table 1).

Juvenoid activity of bisphenol A

Juvenoid hormones are integral signaling molecules in arthropods, including crustaceans, that commonly function in an opposing fashion to ecdysteroid. Thus, juvenoids can be viewed as having anti-ecdysteroid activity. Experiments were performed to determine whether apparent anti-ecdysteroidal activity of bisphenol A could be due to intrinsic juvenoid activity of this compound. The ability of bisphenol A to stimulate the production of male offspring, a highly-specific juvenoid-regulated process in daphnids, was evaluated. Bisphenol A was unable to stimulate the production of male offspring when maternal daphnids were exposed to this compound alone (Fig. 5). However, exposure to bisphenol A along with the juvenoid methyl farnesoate stimulated the production of male offspring to a level greater than that caused by the methyl farnesoate alone (Fig. 5). This effect is characteristic of weak juvenoids such as insect growth regulator methoprene (Fig. 6) and

suggests that the anti-ecdysteroidal activity of bisphenol A could be due to its ability to act as a weak juvenoid hormone.

Effect of bisphenol A on general fecundity

Results from these experiments suggested that bisphenol A could reduce overall fecundity of daphnids by interfering with embryo development resulting in a decrease in the production of viable offspring. This potential was evaluated by continuously exposing maternal daphnids to concentrations of bisphenol A and evaluating the production of viable offspring by these organisms. The number of offspring produced by bisphenol A-exposed daphnids progressively declined with bisphenol A concentration over the range of concentrations that overtly elicited anti-ecdysteroidal activity (2.5-10 mg/L) (Fig. 7). Results suggest that the threshold concentration (i.e. intercept with the dashed control line in Fig. 7) exists at approximately 1.3 mg/L bisphenol A.

Acute:chronic ratio (ACR) of bisphenol A

The ratio of the acute toxicity of a chemical expressed as its EC₅₀ value and its threshold concentration, as measured during full life-cycle exposure, is often used as a measure of the propensity of a chemical to elicit chronic toxicity (Calabrese and Baldwin 1993). The EC₅₀ value for bisphenol A with daphnids was 16 mg/L (95% confidence interval: 15.9-16.4) providing a acute:chronic ratio (16/1.3) of 12. Consistent with this specific mode of reproductive toxicity (anti-ecdysteroidal activity), an ACR of >10 is typically indicative of

chronic toxicity that is elicited by a mechanism distinct from that responsible for acute toxicity (LeBlanc 1997).

DISCUSSION

Results from the present study demonstrate that bisphenol A elicits anti-ecdysteroidal activity with the crustacean *Daphnia magna*. This effect appears to be a consequence of the weak juvenoid activity elicited by the material and may contribute to reproductive toxicity associated with the compound. Bisphenol A was shown to elicit anti-ecdysteroidal activity in cultured insect cells with an EC50 of 23 mg/L (Dinan, Bourne et al. 2001a). In vivo results from the present study were remarkable consistent with this in vitro assessment where anti-ecdysteroidal effects on daphnid molting and embryo development were elicited in the 5 to 10 mg/L range.

Toxicity of bisphenol A to arthropods has previously been reported that is consistent with the anti-ecdysteroidal/juvenoid activity reported in the present study. Molting was delayed in the blood worm (*Chironomus riparius*) at an exposure level of 1 mg/L bisphenol A (Watts, Pascoe et al. 2003). Bisphenol A was reported to inhibit naupliar development of the copepod *Acartia tonsa* with an EC10 of 0.22 mg/L (Anderson, Wollenberger et al. 2001) and mouthpart development in *C. riparius* at 1 mg/L (Watts, Pascoe et al. 2003).

The inability of exogenous 20-hydroxyecdysone to protect against the anti-ecdysteroidal toxicity of bisphenol A argues that the precise mechanism of toxicity does not involve depletion of endogenous ecdysteroid levels or competitive antagonism at the ecdysteroid receptor. We have shown that toxicity involving both of these mechanisms can be ameliorated by co-administration of 20-hydroxyecdysone (Mu and LeBlanc 2002a; Mu and LeBlanc 2002b). This observation stands in contrast to results from cell-free receptor binding assays where bisphenol was shown to compete with the ecdysteroid ponasterone A for binding to the ecdysone receptor (Dinan, Bourne et al. 2001a). This discrepancy may indicate that bisphenol A elicits multiple mechanisms of anti-ecdysteroidal activity and that receptor antagonism is not the predominant mechanism in vivo.

Bisphenol A was shown to exhibit weak juvenoid activity through its ability to act as a male-sex determinant in daphnids. Male sex determination is a juvenoid-regulated process in daphnids (Olmstead and LeBlanc 2002) that is susceptible to the actions of environmental juvenile hormone analogs but not other environmental chemicals evaluated (Olmstead and LeBlanc 2003). Juvenoid hormones have been shown to block the regulatory actions of ecdysteroids in insects. For example, the juvenoid methoprene has been shown to block ecdysteroid-mediated induction of heat shock proteins (Berger, Goudie et al. 1992) and microRNAs (Sempere, Sokol et al. 2003). 20-Hydroxyecdysone can induce (low doses) or suppress (high doses) RNA synthesis in the silk gland of *Galleria mellonella*. However, co-treatment with JHIII can block these actions of ecdysteroids on RNA synthesis (Kodrik and Sehnaal 1994). Juvenile hormones have been shown to suppress ecdysteroid-regulated

molting of the lobster (*Homarus americanus*) larvae (Hertz and Chang 1986) and naupliar development of the copepod (*Acartia tonsa*) (Anderson, Wollenberger et al. 2001). We recently have shown that insecticidal juvenile hormone analogs function as anti-ecdysteroids in a manner comparable to bisphenol A described in the present study (Mu, Hoy et al. 2003). This latter observation lends additional support to the conclusion that the mechanism of anti-ecdysteroidal activity of bisphenol A is due to its juvenoid activity.

Results of the present study provide a mechanism by which bisphenol A can elicit reproductive toxicity to crustaceans. Results further demonstrate that reproductive toxicity of this compound can be detected at exposure concentrations approximating one order-of-magnitude below levels that elicit acute toxicity. While these results are indicative of chronic toxicity of this compound, exposure concentrations at which these effects are elicited remain significantly above expected environmental levels. In a survey of 139 streams across the United States that were downstream of significant urbanization or agriculture, bisphenol A was detected in 41% of the sites. The median concentration detected was 0.14 µg/L and the maximum concentration measured was 12 µg/L (Kolpin, Furlong et al. 2002). The threshold concentration for reproductive toxicity of 1.3 mg/L determined in the present study suggests that this compound poses no significant risk of toxicity due to anti-ecdysteroidal activity at levels expected to occur in the environment.

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Table 1. Susceptibility of variously aged embryos to the developmental toxicity of bisphenol

A. Age denotes the length of time that embryos were allowed to develop in the brood chamber of maternal organisms following transfer from the ovaries. Embryos were removed at the designated time and allowed to continue development ex vivo while exposed to the indicated concentration of bisphenol A. The incidence of developmental abnormalities was assessed upon completion of development among the respective control embryos (~72 hrs.). Each treatment consisted of 19-36 embryos.

Experiment	Embryo Age (hr)	Bisphenol A (mg/L)	Developmental Abnormalities (%)
1	6.5	0	0
		2.5	56
2	8.0	0	0
		10	35
3	8.0	0	0
		5.0	17
		10	21
4	12	0	0
		5.0	0
		10	0
5	18	0	0
		20	54

Figure Legend

Figure 1. Intermolt duration between the first and second molt of neonatal daphnids exposed to concentrations of bisphenol A. Each data point represents an individual daphnid. The data were fit to a two segmented line with associated 95% confidence intervals. Mean ($\pm$ standard deviation) performance of 10 unexposed (control) daphnids is depicted by the open square.

Figure 2. Percentage neonates with developmental abnormalities during exposure to concentrations of bisphenol A. Each data point represents the percentage abnormal neonates produced by a single maternal daphnid exposed to the indicated concentration of bisphenol A. Mean ($\pm$ standard deviation) performance of 10 unexposed (control) daphnids is depicted by the open square.

Figure 3. . Influence of 20-hydroxyecdysone (20E) exposure on delayed molting caused by bisphenol A (BPA). Neonatal daphnids (<1 hr old) were individually exposed to 9.0 mg/L bisphenol A alone or in combination with concentrations of 20E. The duration of the first intermolt period was measured as described in the methods. Data are presented as mean and standard deviation (n=8). An asterisk indicates a significant difference from the control at $P \leq 0.05$ (ANOVA, Dunnett's t test).

Figure 4. Influence of 20-hydroxyecdysone (20E) exposure on developmental abnormalities caused by bisphenol A (BPA). Embryos were removed from maternal daphnids 6.5 hrs (A)

or 18 hrs (B) after deposition from the ovaries to the brood chamber and individually exposed to either 2.5 mg/L (A) or 20 mg/L (B) bisphenol A in the presence or absence of 20-hydroxyecdysone. The incidence of developmental abnormalities was assessed upon completion of development among control embryos (~80 hrs). Each treatment consisted of 23-24 individual embryos.

Figure 5. Incidence of male-sex determination among offspring produced by maternal daphnids exposed to methyl farnesoate (MF), bisphenol A (BPA), or combinations thereof. Maternal daphnids (7-10 per treatment) were exposed to the chemicals, as indicated, through a complete period of oocyte/embryo maturation. Resulting neonates were sexed upon release from the maternal daphnids and the percentage broods that contained male offspring were determined for each treatment. The 'n' denotes the number of times the experiment was repeated. Data are presented as the mean and standard error of the replicate experiments.

Figure 6. Incidence of male-sex determination among offspring produced by maternal daphnids exposed to methyl farnesoate (MF), methoprene (MP), or combinations thereof. Maternal daphnids (8-11 per treatment) were exposed to the chemicals, as indicated, through a complete period of oocyte/embryo maturation. Resulting neonates were sexed upon release from the maternal daphnids and the percentage broods that contained male offspring were determined for each treatment.

Figure 7. Total offspring produced among daphnids continuously exposed to concentrations of bisphenol A for 21 days. Each data points represents the total offspring produced by a single maternal daphnid. Dashed lines represent the mean and standard deviation number of offspring produced by 10 control daphnids. Data were fit to a second-order regression line.

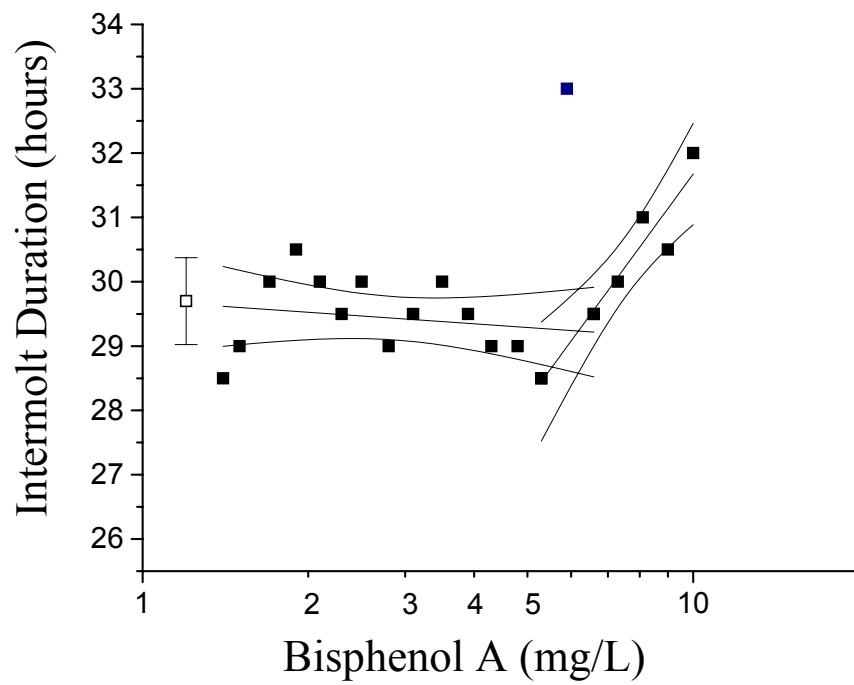


Figure 1

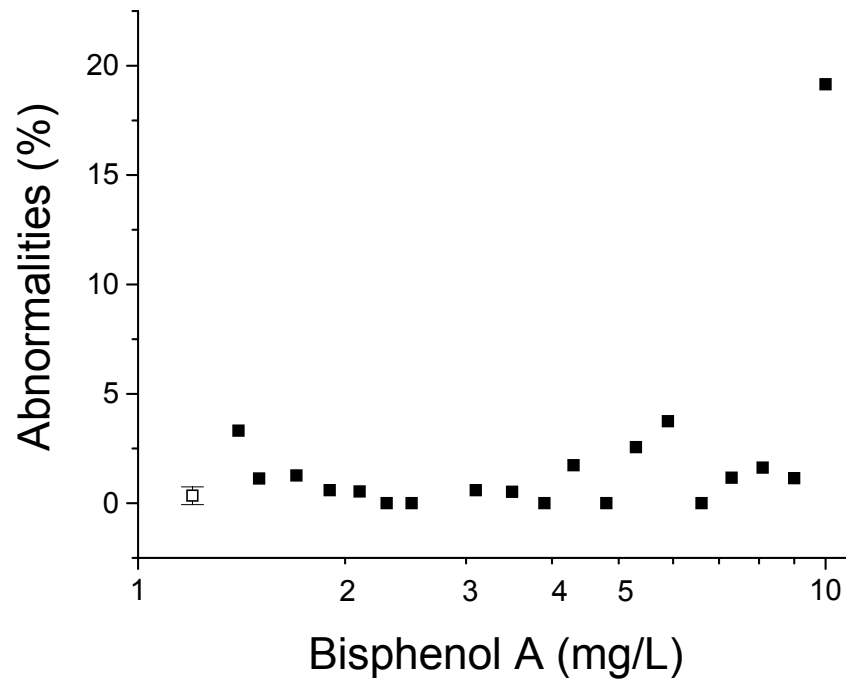


Figure 2

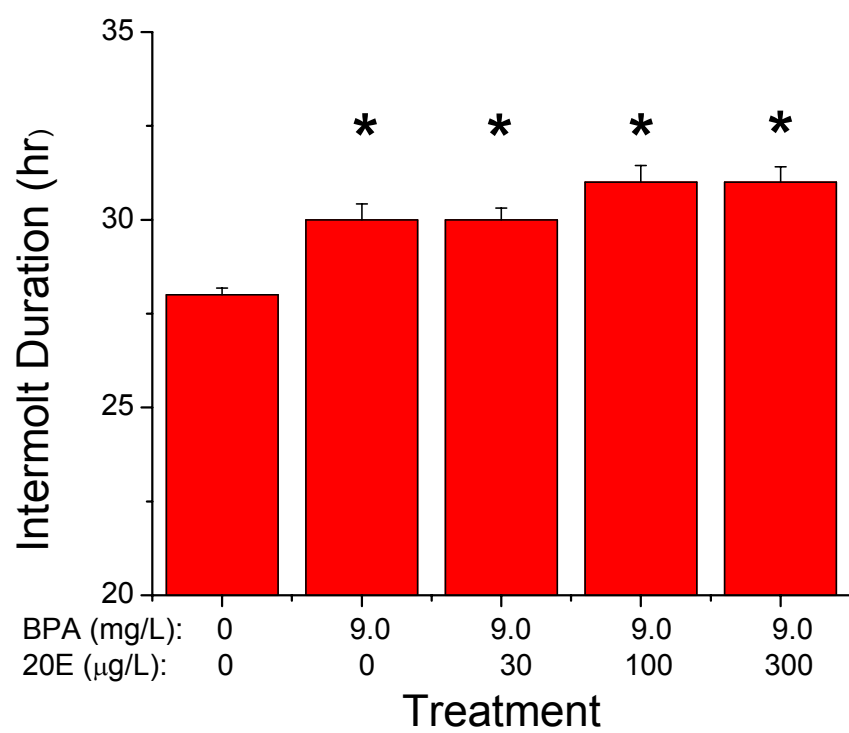


Figure 3

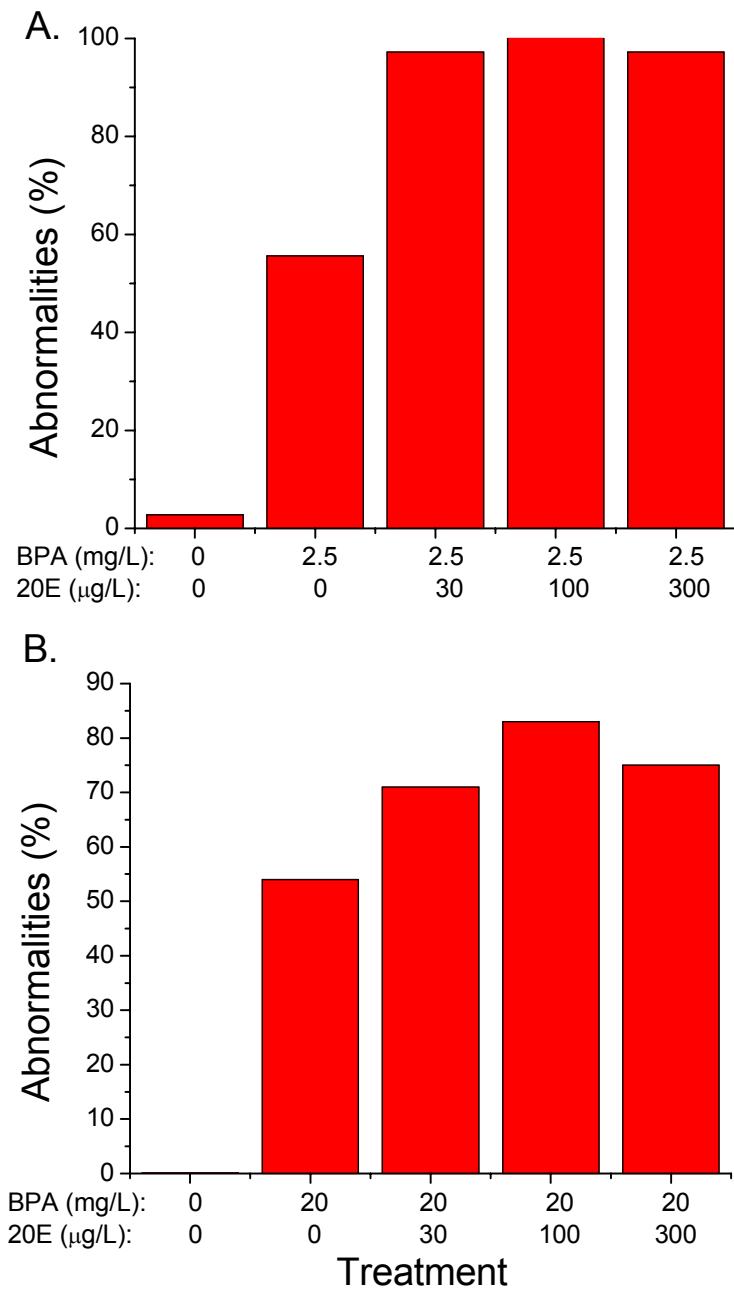


Figure 4

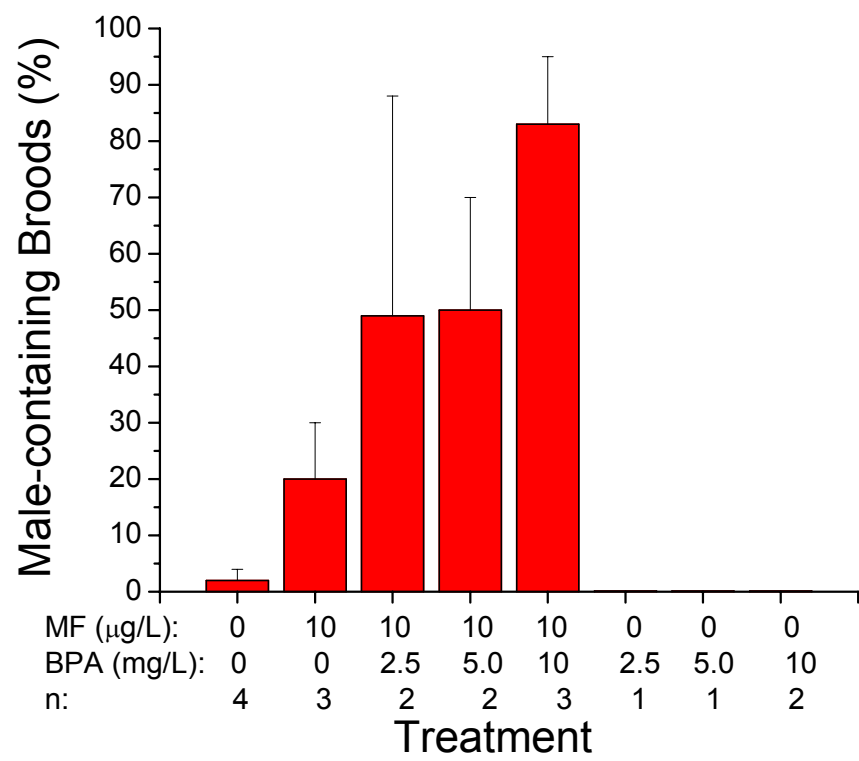


Figure 5

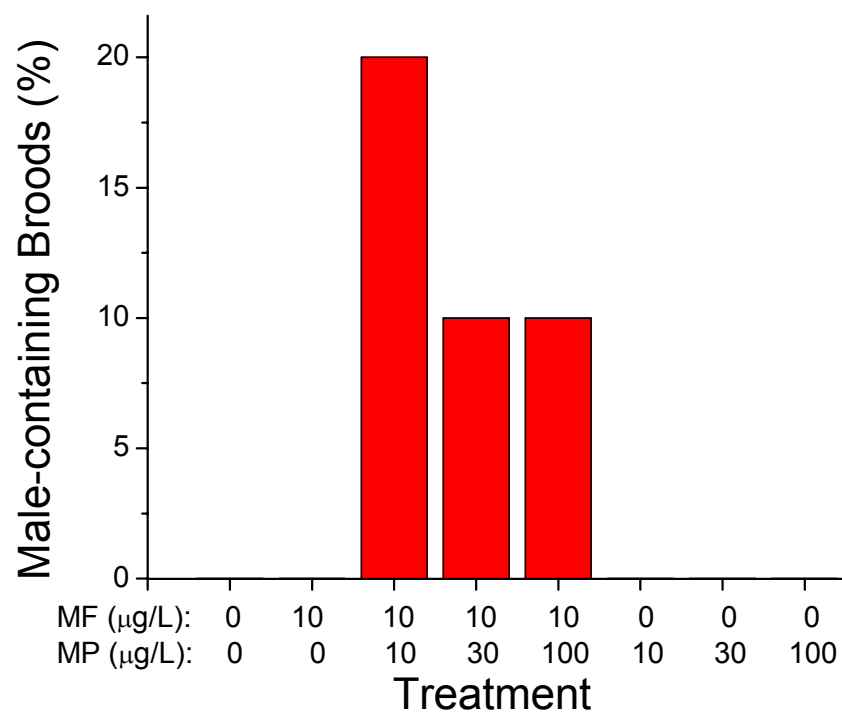


Figure 6

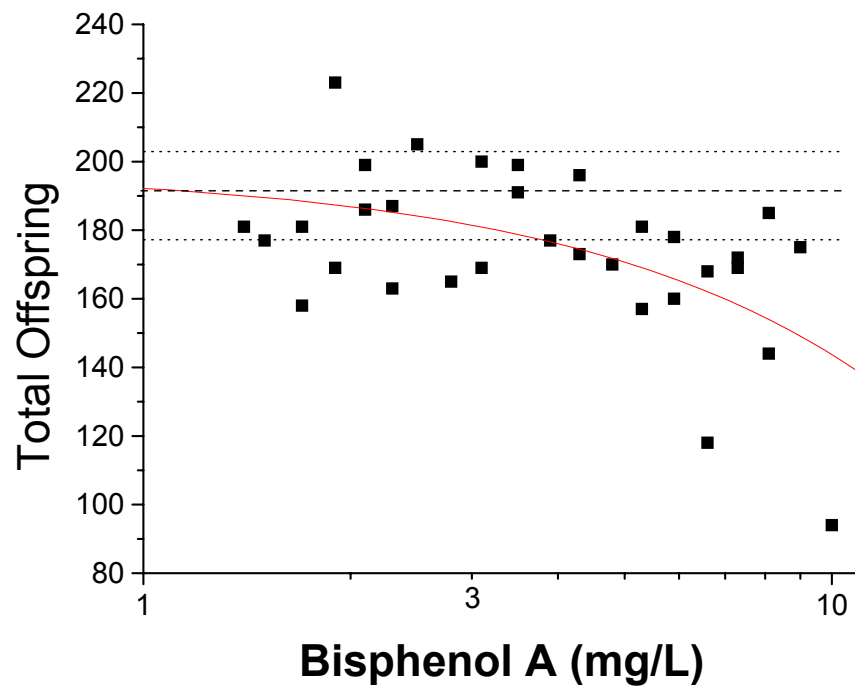


Figure 7

SUMMARY AND CONCLUSION

The focus of this research was to investigate the mechanisms by which environmental chemicals may disrupt ecdysteroid-regulated processes in crustaceans and the consequences of these perturbations. We demonstrated that ecdysteroids are critical to normal crustacean embryo development. As a consequence, environmental anti-ecdysteroids could interfere with the normal embryo development and elicit developmental toxicity. Three mechanisms, lowered endogenous ecdysone levels, ecdysone receptor antagonism, and decreased gene expression of the ecdysone receptor, were identified by which environmental chemicals can elicit their anti-ecdysteroidal activity in *Daphnia magna*. All of these anti-ecdysteroidal activities associated with exogenous chemicals interfered with embryo development, reduced embryo viability, and compromised overall fecundity.

The agricultural fungicide fenarimol was selected to evaluate the role of ecdysteroids in crustacean embryo development and the susceptibility of the developing embryo to the anti-ecdysteroidal properties of environmental chemicals. Fenarimol is known to inhibit cytochrome P450 enzymes (Williams et al., 2000) and we accordingly hypothesized that it also could interfere with ecdysone synthesis in daphnids.

Fenarimol was shown to exhibit anti-ecdysteroidal activity to the crustacean *Daphnia magna* by lowering endogenous ecdysone levels and delaying molting in a concentration-dependent fashion that was abrogated by co-exposure to exogenous 20-hydroxyecdysone.

Exposure of either gravid maternal organisms or isolated embryos to fenarimol resulted in embryo abnormalities ranging from early partial developmental arrest to incomplete development of antennae and shell spines. Developmental abnormalities were associated with suppressed ecdysone levels in the embryos and the abnormalities could be prevented by co-exposure to 20-hydroxyecdysone. Developmental abnormalities caused by the antiecdysteroid fenarimol were associated with reduced fecundity of the parental organisms. These results demonstrate that ecdysteroids are critical to normal crustacean embryo development and environmental anti-ecdysteroids can disrupt normal embryo development and compromise the production of viable offspring.

We have observed that testosterone causes developmental arrest in embryos of the crustacean *Daphnia magna* at exposure concentrations well below those that are overtly toxic to the maternal organisms (LeBlanc et al., 2000). Considering that fenarimol elicits embryo toxicity similar to testosterone, and that this toxicity of fenarimol is due to anti-ecdysteroidal activity of the fungicide, we considered the possibility that the embryotoxicity of testosterone might be due to anti-ecdysteroidal activity associated with testosterone.

The effect of testosterone on molt frequency of early instar daphnids was first evaluated to determine whether testosterone interfered with this ecdysteroid-regulated process. Molt frequency was delayed by exposure to testosterone and this effect was mitigated by co-exposure to the ecdysteroid 20-hydroxyecdysone. Testosterone exposure concentrations that interfered with molting also elicited developmental abnormalities among

neonatal organisms produced by maternal organisms that were continuously exposed to testosterone or among embryos that were removed from unexposed mothers and exposed directly to the hormone. Embryos were significantly protected against the developmental toxicity of testosterone by co-exposure to 20-hydroxyecdysone. Take together, these results demonstrated that the embryo toxicity of testosterone to daphnids is due largely to its ability to interfere with ecdysteroid control of development. Experiments next were conducted to determine whether testosterone interfered with ecdysteroidal activity by acting as an ecdysone receptor antagonist or by reducing endogenous ecdysone levels. Testosterone significantly antagonized the action of 20-hydroxyecdysone in an ecdysone-responsive cell line. Testosterone had no discernable effect on endogenous ecdysone levels in daphnids. These results demonstrated that testosterone elicits embryo toxicity to daphnids by interfering with ecdysteroid activity, and ecdysteroid receptor antagonism could be one mechanism by which testosterone elicits these effects.

Endocrine toxicants can interfere with hormone signaling through various mechanisms. Some of these mechanisms are interrelated in a manner that might result in synergistic interactions (Kortenkamp and Altenburger, 1998). Based upon results of the first two studies, we concluded that fenarimol is an ecdysteroid synthesis inhibitor and testosterone elicits anti-ecdysteroidal activity by functioning as ecdysteroid receptor antagonist. Next, we tested the hypothesis that a hormone synthesis inhibitor and a hormone receptor antagonist would synergize to yield greater toxicity than that predicted from a model of independent joint action. This hypothesis was tested by assessing the effect of the

ecdysteroid synthesis inhibitor fenarimol and the ecdysteroid receptor antagonist testosterone on ecdysteroid- regulated embryo development in the crustacean *Daphnia magna*. Both compounds were individually characterized for effects on the development of isolated embryos. Fenarimol caused late developmental abnormalities, consistent with its effect on offspring-derived ecdysone in the maturing embryo. Testosterone interfered with both early and late development of embryos, consistent with its ability to inhibit ecdysone provided by maternal transfer (responsible for early developmental events) or de novo ecdysone synthesis (responsible for late developmental events). We predicted that, by decreasing endogenous levels of hormone, fenarimol would enhance the likelihood of testosterone binding to and inhibiting the ecdysone receptor. Indeed, fenarimol enhanced the toxicity of testosterone; while, testosterone had no effect on the toxicity of fenarimol. Algorithms were developed to predict the toxicity of combinations of these two compounds based upon independent joint action (IJA) alone, as well as IJA with fenarimol-on-testosterone synergy (IJA+SYN). The IJA+SYN model was highly predictive of the experimentally-determined combined effects of the two compounds. These results demonstrate that endocrine toxicants can synergize, and this synergy can be accurately predicted.

Juvenile hormone analogs (JHAs) have been widely used as insect pest control agents, but their effects on non-target crustacean species are largely unknown (LeBlanc et al., 1999). In following experiments, we tested the hypothesis that juvenoids can regulate ecdysteroid activity in the crustacean (*Daphnia magna*) in an antagonistic fashion. The crustacean juvenoid hormone methyl farnesoate and the insecticidal JHAs pyriproxyfen and

fenoxycarb all disrupted ecdysteroid-regulated aspects of embryo development in daphnids. In vitro exposure of ecdysteroid-responsive cells to 20-hydroxyecdysone reduced cell proliferation and increased mRNA levels of the ecdysone receptor and its partner protein ultraspiracle. Co-treatment of cells with the juvenoid pyriproxyfen attenuated all of the ecdysteroid mediated responses. While juvenoids functioned as anti-ecdysteroids both in vivo and in cultured cells, 20-hydroxyecdysone showed no evidence of acting as an anti-juvenoid. The combined effects of pyriproxyfen and the anti-ecdysteroid fenarimol on embryo development were evaluated in an effort to discern whether these compounds acted in an additive or synergistic manner. The combined action of the juvenoid and anti-ecdysteroid conformed to a model of synergy. These results demonstrated that juvenoids elicit anti-ecdysteroidal activity in a crustacean through a novel mechanism. A model involving receptor partner deprivation is proposed.

Bisphenol A is a key industrial chemical used in the manufacture of polycarbonate plastics, epoxy resins and other products. Several recent reports ascribe toxicological properties to this compound that have been attributed the disruption of endocrine-related processes. In the last part of my dissertation research, the toxicity of bisphenol A was definitively characterized in the *Daphnia magna* in an effort to discern whether this compound may elicit endocrine-related toxicity in an invertebrate species at environmentally-relevant concentrations. The ability of bisphenol A to interfere with two ecdysteroid-dependent physiological processes – molting and embryo development - was evaluated. Bisphenol A elicited anti-ecdysteroidal activity as indicated by its prolongation of the

intermolt period and interference with embryo development. However, neither effect was abrogated by co-exposure to 20-hydroxyecdysone. Thus, the apparent anti-ecdysteroidal activity of this compound was neither due to reduced availability of endogenous ecdysteroid nor due to ecdysteroid receptor antagonism. Juvenoid hormones are known to function as anti-ecdysteroid, therefore, the ability of bisphenol A to elicit juvenoid activity was evaluated. Bisphenol A did not elicit intrinsic juvenoid activity, but did increase the production of male offspring – a juvenoid regulated process – when co-administered with the juvenoid hormone methyl farnesoate. This effect in the presence of a more potent juvenoid is characteristic of a weak juvenoid. A definitive assessment of the effects of bisphenol A on the reproductive capacity of daphnids revealed a linear concentration-response relationship that extended at least one order-of-magnitude below exposure levels that were overtly toxic to the maternal organisms. These results demonstrate that bisphenol A is chronically toxic to daphnids, probably through its anti-ecdysteroidal/juvenoid activity. However, effects are elicited at levels that are not likely to pose an environmental threat.

Overall, results of this study provide conclusive evidence that diverse environmental chemicals can elicit anti-ecdysteroidal activity in crustaceans through multiple mechanisms. As a consequence of this activity, these chemicals interfere with embryo development, reduce embryo viability, and compromise overall fecundity. Such effects of environmental contaminants should be considered when evaluating causes of population decline among economically-important crustacean populations.

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