

OLMSTEAD, ALLEN W. Environmental Toxicant Effects on Sexual Reproduction in *Daphnia magna* (under the direction of Gerald A. LeBlanc)

The freshwater zooplankton, *Daphnia magna* alternates between asexual and sexual reproductive strategies. Asexual reproduction allows these animals to rapidly exploit abundant resources and favorable environmental conditions, while sexual reproduction produces developmentally-arrested eggs that can withstand extreme environmental conditions. While asexual reproduction is widely studied in ecotoxicological studies, sexual reproduction has been largely ignored and the extent to which xenobiotics can interfere with this process is not known. I hypothesized that certain environmental toxicants can interfere with sexual reproduction in *Daphnia magna* by disrupting the endocrine regulation of this process. This hypothesis was tested by: 1) demonstrating that multiple aspects of daphnid sexual reproduction could be affected by chemical exposure, 2) elucidating the endocrine processes responsible for regulating an early component of the sexual reproductive phase, and 3) evaluating the susceptibility of this process to disruption by environmental chemicals.

Several components of daphnid sexual reproduction were shown to be affected by chemical exposure. The development of secondary sex characteristics in female (abdominal process) and male daphnids (elongated first antennae) was characterized and used as endpoints in chemical exposures. Juvenile female daphnids exposed to the estrogen, diethylstilbesterol and to the juvenile hormone mimic, methoprene showed increased development of the abdominal process when compared to untreated animals. Exposure of juvenile male daphnids to the androgen, androstenedione stimulated the growth of the first

antennae. An assay was developed that utilized small daphnid populations to assess the effects of xenobiotic exposure on male and resting egg production. The juvenile hormone mimic, methoprene, was found to stimulate male production in daphnids as well as shift the reproductive output from neonatal production to the production of resting eggs.

Further studies focused on elucidating the mechanism by which methoprene increased male production. The crustacean terpenoid hormone, methyl farnesoate, was found to program eggs in the ovaries to develop into males during neonatal development. This effect only occurred when eggs were exposed during a specific period of ovarian maturation just prior to their release into the brood chamber. Methoprene, a known juvenile hormone agonist in insects, stimulated male production in a similar fashion. Other chemicals with differing mechanisms of action did not alter sex ratios in exposed populations using the same experimental design employed with methoprene. Pyriproxyfen, another juvenile hormone mimic, however, did alter sex ratios in both population and individual exposures with a greater potency than methoprene or methyl farnesoate. Binary mixture studies with these insecticides and methyl farnesoate more closely conformed to a concentration addition model than an independent joint action model suggesting that these three chemicals all exert their effects on sex determination via the same target. These results support a mechanism of action of methyl farnesoate disruption for the insecticide methoprene and suggest that increased male production is a unique effect of exposure to chemicals with the ability to mimic methyl farnesoate.

This research demonstrates that sexual reproduction in daphnids can be altered by xenobiotic exposure which more fully incorporates the complete life cycle of daphnids into toxicity studies. The characterization of the role of methyl farnesoate in daphnid sex determination improves the basic understanding of crustacean endocrinology and the function of methyl farnesoate in these animals. A model for methyl farnesoate disruption can be generated from this work that allows us to better predict and detect chemicals that act as endocrine disruptors in crustaceans.

**ENVIRONMENTAL TOXICANT EFFECTS ON SEXUAL REPRODUCTION IN
*DAPHNIA MAGNA***

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BIOGRAPHY

I was born July 26, 1974 in New Bern, North Carolina where the waters of the Trent river empty into those of the Neuse. My youth was spent hunting, fishing, and trapping in the swamps and lowlands of the coast. I left home at age 15 to attend the North Carolina School of Science and Mathematics where I graduated in 1992. As a high school senior I became interested in environmental issues and began an afternoon mentorship at the Environmental Protection Agency in Research Triangle Park where I helped in experiments measuring levels of atmospheric pollutants. I studied as an undergraduate at North Carolina State University where I earned Bachelor of Science degrees in Chemistry, Biochemistry, and Zoology in 1996-7. In the evenings I worked my way from dishwasher to assistant manager of a local restaurant. The summer after my undergraduate years, I spent assisting my dad raise and harvest coastal Bermuda hay and met my wife, Bethany, who I married on April 1, 2000. I subsequently returned to North Carolina State University to pursue a graduate degree in Toxicology with research focusing on the aquatic environment.

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INTRODUCTION

Environmental endocrine disruption has moved to the forefront of toxicology in recent years. The ability of environmental contaminants to disrupt endocrine-regulated pathways in wildlife species has been well documented (Tyler *et al.*, 1998; Vos *et al.*, 2000) with examples in fish (Folmar *et al.*, 1996), reptiles (Crain and Guillette, 1998), amphibians (Hayes *et al.*, 2002), and snails (Matthiessen and Gibbs, 1998). While the majority of this work has focused on vertebrate species, the invertebrates should not be forgotten. With the exception of imposex in marine gastropods, there is a significant lack of invertebrate models for endocrine disruption. Research into invertebrate endocrine disruption is needed to fill in these gaps so that we can identify chemicals with the ability to alter the endocrinology of these organisms.

The potential for endocrine disruption in crustacean species is not well known, due in part to a lack of basic understanding of the endocrinology of crustaceans. The need for understanding of the potential for endocrine disruption in crustaceans stems from the commercial and environmental importance of these animals. Lobsters, crabs, and shrimp are all large-scale commercial fisheries important to the economies of coastal regions. For the years, 1999 and 2000 in the United States, these fisheries were valued at 1.4 billion dollars and accounted for just over 40 percent of the total value of all U.S. commercial fisheries each year (National Oceanic and Atmospheric Administration, 2001). Reductions in these fisheries caused by toxicant exposure to crustacean populations could severely impact these coastal communities. Smaller crustaceans such as copepods, cirripedes, and cladocerans are important as primary and secondary consumers in aquatic environments (Moss, 1998). This

can be seen in the technique of biomanipulation which is used to reduce algal biomass in shallow lakes by increasing zooplankton populations, which in turn puts greater grazing pressure on the algae. These zooplankton are key links in the transfer of energy up trophic levels and provide a mechanism for the recycling of nutrients for plant use (Moss, 1998). Toxic insults to these zooplankton populations could result in alterations throughout the whole aquatic ecosystem.

The water flea, *Daphnia magna*, is the crustacean used in the following research to develop a model for crustacean endocrine disruption. The crustacean genus *Daphnia* is made up of relatively large freshwater zooplankton, common in lakes and ponds throughout the world (Moss, 1998). Daphnids have been used extensively in ecotoxicological studies and a large database of information regarding the responses of these organisms to toxicant exposure can be found in the literature. A complete life cycle study with daphnids, following individuals from neonates through a full reproductive period, can be performed in about 3 weeks allowing for numerous studies to be performed within a relatively short length of time. Also, *Daphnia magna* reproduce by cyclical parthenogenesis, which allows for study of both asexual and sexual reproductive strategies.

Cyclical parthenogenesis is a complex reproductive strategy found in certain invertebrate species such as rotifers, aphids, and cladocerans (Korpelainen, 1990). During periods of favorable environmental conditions, a given population of cyclical parthenogens reproduce asexually with females producing all female offspring. This allows the population to expand clonally and exploit abundant resources faster than those species reproducing

sexually (Cuellar, 1977). In response to certain environmental cues that signal a degradation in the conditions of the habitat, the population switches to sexual reproduction, the final product of which is eggs in a state of diapause. In daphnids, the first step in this switch from asexual to sexual reproduction is the production of males, which mature and mate with females, resulting in the production of developmentally arrested eggs (Hebert, 1978). In daphnids these resting eggs are enclosed in a modified portion of the mother's carapace called an ephippium. These ephippia are highly pigmented and protect the eggs from such harmful conditions as freezing, desiccation (Hebert, 1978), or even passage through the digestive tract of a predator (Mellors, 1975). Resting eggs can survive extreme environmental conditions that may eliminate the adult population and then re-initiate development and grow into adult females when favorable conditions return. These females mature and initiate parthenogenesis to complete the reproductive cycle in a daphnid population.

Very little attention has been paid to the ability of chemicals to interfere with daphnid sexual reproduction, while parthenogenic reproduction has been studied extensively (Landis and Yu, 1995). The importance of sexual reproduction to a daphnid population should not be overlooked. Sexual reproduction allows for these populations to survive periods of extreme environmental conditions, particularly those in which the adults are unable to survive such as the drying out or freezing of a pond. When dried out these ephippia are light and can be dispersed by the wind allowing for colonization of new habitats (Pennak, 1953). Sexual reproduction also allows for genetic recombination and gene flow to occur in a population.

In all species of cyclical parthenogens, the switch from asexual to sexual reproduction is triggered by environmental cues (Korpelainen, 1990). In daphnids these include increased population density, reduced food levels or quality, and shortened daylengths (Carvalho and Hughes, 1983; Kleiven *et al.*, 1992; Stross and Hill, 1965). Sensitivity to these triggers vary among species (Deng, 1996) and can even vary among clones of the same species (Ferrari and Hebert, 1982; Larsson, 1991). Often, the collective pressure of several of these stimuli is necessary for the switch to sexual reproduction to occur (Kleiven *et al.*, 1992; Korpelainen, 1989). While the environmental cues that induce sexual reproduction have been studied in daphnids, the hormonal pathways that cause this switch in reproductive strategy have not been examined and are not understood.

In aphids, an insect species that also reproduces by cyclical parthenogenesis, the switch from asexual to sexual reproduction is modulated by the terpenoid, Juvenile Hormone III (Nijhout, 1998). In their natural habitat, shortening of the daylength and a lowering of the temperature causes the production of male offspring, the first step in the sexual reproductive process for aphids. Exposure to kinoprene, a juvenile hormone mimic, causes a reduction in male progeny production (Mittler *et al.*, 1979). Exposure of insects to precocene causes the destruction of the corpus allatum, the source of juvenile hormones in insects (Retnakaran *et al.*, 1985). Young adult female aphids treated with precocene have reduced juvenile hormone titers and produce more males than controls (Hales and Mittler, 1983). This effect can be rescued by the subsequent application of the juvenile hormone mimic, kinoprene (Hales and Mittler, 1988). These experiments demonstrate that juvenile hormones inhibit

male production in aphids and likely negatively impacts the ability of these organisms to enter the sexual reproductive phase.

The endocrinologies of insects and crustaceans are remarkably similar. Both groups of organisms rely heavily on neuro-endocrine structures and peptide hormones and both use two types of lipidic hormones, ecdysones and juvenile hormones (LaFont, 2000). While none of the specific insect juvenile hormones are found in crustaceans, a related terpenoid, methyl farnesoate, is believed to be a crustacean hormone (Homola and Chang, 1997; Laufer and Biggers, 2001). Methyl farnesoate is in fact the precursor to Juvenile Hormone III in insect synthetic pathways, with conversion from methyl farnesoate to Juvenile Hormone III occurring through the epoxidation of one of methyl farnesoate's double bonds (Wang *et al.*, 1994). In decapod crustaceans methyl farnesoate is produced by the mandibular organ and is under negative regulation by the sinus gland (Laufer and Biggers, 2001). Methyl farnesoate in crustaceans has been implicated as having a role in reproduction (Borst *et al.*, 1987; Homola and Chang, 1997; Laufer *et al.*, 1993; Laufer and Biggers, 2001) and juvenile development (Homola and Chang, 1997; Laufer and Biggers, 2001). The presence of methyl farnesoate has widely been reported in various decapod species. Methyl farnesoate also has been reported to occur in the barnacle and brine shrimp (Laufer and Biggers, 2001). Considering the similarities in insect and crustacean endocrinology, it is possible that juvenoids are involved in the regulation of daphnid sexual reproduction. Juvenile hormones are also involved in pathways leading to larval diapause in insects. The diapause state in the European corn borer, *Ostrinia nubilalis*, larva is initiated and maintained by the presence of

high titers of Juvenile Hormone (Yagi and Akaike, 1976). It is likely, then, that methyl farnesoate plays a similar role in diapausing crustaceans.

There is a unique set of insecticides designed to target juvenile hormone regulated processes by acting as terpenoid agonists (Retnakaran *et al.*, 1985). Methoprene is the most well-known of these chemicals and is applied directly to the aquatic environment where it inhibits the development of mosquito larva. In normal mosquito development Juvenile Hormone III titers are kept high until the fourth instar (Dhadialla *et al.*, 1998; Retnakaran *et al.*, 1985). During this instar titers are reduced, triggering the metamorphosis of the larva into the adult form at the next molt. Larva exposed to methoprene never experience this drop in juvenile hormone activity due to the agonistic properties of methoprene. The larva continues to molt and grow larger but never metamorphosizes into the adult pest form. These juvenile hormone mimicking insecticides make ideal candidates for assessing the potential for disruption of terpenoid-regulated processes in crustaceans. This form of endocrine disruption is particularly relevant in that these insecticides are often applied directly to the aquatic environment ensuring exposure of crustaceans.

My hypothesis is that daphnid sexual reproduction can be altered by exposure to toxicants by a mechanism of endocrine disruption. To assess this hypothesis, the susceptibility of aspects of sexual reproduction, namely development of secondary sex characteristics, male production, and the shift from parthenogenesis to sexual reproduction, to xenobiotic exposure was evaluated. Based on the results from these experiments, sex determination was selected for more detailed studies to elucidate a mechanism of action for

the observed effects. The basic endocrinology of daphnids sex determination was studied and a model for sex determination in daphnids was constructed whereby a certain level of terpenoid hormone activity during ovarian development causes the resultant offspring to develop into males. Experiments were then conducted to show that exposure to juvenile hormone-mimicking insecticides during this window causes a shift in offspring sex ratio from females to males by agonism of methyl farnesoate pathways. Experiments with other chemicals lacking juvenile hormone activity failed to induce the production of males suggesting that this endpoint is particularly selective for juvenile hormone mimics. Further studies with binary mixtures add support for a mechanism of methyl farnesoate disruption of these insecticides. The following research assesses the potential for chemicals to affect daphnid sexual reproduction and develops a model for the study of endocrine disruption of the methyl farnesoate hormonal system in crustaceans.

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**Effects of Endocrine Active Chemicals on the
Development of Sex Characteristics of *Daphnia magna***

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ABSTRACT

Standard reproductive assays with daphnids involve parthenogenetically reproducing females and exclude the assessment of effects on sexual reproduction. The goals of this study were to characterize sexual differentiation of male and female daphnids (*Daphnia magna*) and to evaluate whether exposure to putative endocrine-disrupting chemicals may perturb the development of sex characteristics. Anatomical sex differences that developed during maturation in males included elongated first antennae and morphological alterations in the head capsule and carapace edge. Reproductive maturation in females was associated with the development of a brood chamber and abdominal process. Alterations in the growth rates of the first antennae of males and the abdominal process of females were used to evaluate the effects of chemical exposure on the development of these sex characteristics during maturation. Exposure of female daphnids to the nonsteroidal vertebrate estrogen diethylstilbesterol (DES) and the insect juvenile hormone analog methoprene at concentrations as low as 3.0 μM and 0.080 μM , respectively, stimulated development of the abdominal process. Exposure of males to the steroidal vertebrate androgen androstenedione (≥ 6.0 μM) stimulated development of the first antennae. These results demonstrate that the development of secondary sex characteristics in daphnids can be altered by chemical exposure.

INTRODUCTION

In 1996, the U.S. Congress mandated that the U.S. Environmental Protection Agency develop and implement screening and testing methods for the evaluation of toxicity associated with endocrine-disrupting chemicals. In response, various testing approaches were considered that would serve to detect endocrine toxicity [1]. A battery of testing methods have been in place for decades, for the evaluation of chemical toxicity as required under legislation such as the Federal Insecticide and Rodenticide Act and the Toxic Substances Control Act [2]. An expeditious means of evaluating the endocrine-disrupting potential of chemicals would be to incorporate endocrine-relevant endpoints into these existing testing protocols.

Cladocerans, including *Daphnia magna*, have been used extensively as representative freshwater invertebrates when evaluating the toxicity of chemicals that pose some risk for contaminating the environment through manufacture, use, or disposal [3]. *D. magna* populations reproduce by cyclic parthenogenesis involving both sexual and asexual reproduction [4, 5]. Laboratory cultures of daphnids are typically maintained in the parthenogenetic state. Further, life cycle toxicity assessments typically include only evaluations of the effects of a chemical on parthenogenetic reproduction [6]. Evidence of sexual reproduction in cultures for toxicity tests has typically been used as an indicator of poor culturing or testing techniques [3].

Sexual reproduction in daphnids is critical to population viability because fertilized eggs (resting eggs) can sustain freezing or drying and thus provide a means of repopulation

in new, more favorable environments or following environmental adversity [4, 7]. Sexual reproduction also provides a means for reshuffling of the gene pool and increasing genetic diversity in a population [4]. Sexual reproduction includes many putative endocrine regulated processes that are not evaluated when assessing toxicity only to parthenogenetically reproducing organisms. These include: a) sex ratios of offspring, b) development of secondary sex characteristics, c) fertility, and d) hatching success of resting eggs.

The development of sexual characteristics has been shown to be affected by chemical insult in a number of different animals. Alligators from Lake Apopka, a site contaminated with the organochlorine pesticides dicofol and DDT, were found to have diminutive phalli, poorly organized testes, and significantly lower plasma testosterone concentrations when compared to a reference lake, Lake Woodruff [8]. 4-Tert-octylphenol stimulated vitellogenin production and caused reproductive impairment in male Japanese medaka [9].

Invertebrates are likewise susceptible to toxicant insult resulting in deviations from normal sexual differentiation. The most well documented account is that of exposure to tributyltin resulting in the formation of a penis in female gastropods, a condition known as imposex [10]. High incidences of intersexuality in populations of copepods were reported in areas receiving sewage discharge [11]. Short-term exposure of neonatal daphnids to t-amyl phenol during a critical window of development has been observed to cause females to develop certain male secondary sex characteristics [12]. Exposure of daphnid populations to atrazine in the laboratory has been shown to alter the sex ratio of the offspring [13].

The specific objectives of this study were to identify anatomical traits that are characteristic of normal sexual differentiation in daphnids and then evaluate the susceptibility of these parameters to disruption by exposure to a hormonally active stilbene, a terpenoid, and a steroid. The goal of this study was to identify endpoints of sexual maturation in daphnids that are susceptible to perturbation from chemical exposure and could be readily monitored during standard reproductive toxicity tests.

MATERIALS AND METHODS

Daphnia magna

Daphnids were cultured in medium as described previously [14]. Daphnids were provided a diet of green algae (*Selenastrum capricornutum*) supplemented with Tetrafin (Pet International, Chester Hill, New South Wales, Australia) fish food. Algae were cultured in Bold's basal medium [15]. The fish food supplement was prepared by homogenizing 10 g of fish food in a blender with 1 L of water for 10 minutes. Solids were settled out overnight and the resulting supernatant was used. Aliquots of the supernatant were dried at 100°C to determine the suspended solids content. Daphnid cultures (1 L medium containing 40 daphnids) were provided 1.4×10^8 cells of algae twice daily and 4 mg (dry weight) of fish food suspension twice daily. Daphnids were transferred to new medium and offspring removed three times weekly. Cultures and all experiments were maintained at 20°C with a 16 hour photoperiod. These culture conditions maintained the daphnids in the parthenogenic reproductive stage.

Stimulation of male production

Male production was stimulated by culturing 40 female daphnids (<24 hours old) in 1 L of medium. Daphnids were provided 7×10^7 cells of algae twice daily from day 0 to day 4. Starting on day 5, about the time that ovaries are maturing, the amount of algae added to the cultures was reduced to 3.5×10^7 cells twice daily. Neonates were removed daily, sex was determined by the length of the first antennae under 10X magnification (the first antennae of neonatal males is about ten times longer than that of females), and each sex was cultured separately.

Characterization of Sexual Differentiation

Daphnids (<24 hours old) were raised in 250 ml beakers containing 200 ml of daphnid media. For the evaluation of female daphnids, each beaker contained six daphnids and was provided 1.8×10^7 cells of algae twice daily. For the evaluation of males, each beaker contained twelve daphnids and was provided 1.0×10^8 cells of algae twice daily. Females and males were evaluated under different conditions due to differences in size. The medium was renewed every three days with the experiments ending after ten days.

The daphnids from one beaker were examined microscopically every day to assess sexual development. Daphnids were placed on a glass microscope slide, immobilized by removing the medium from the slide, and anatomical development was noted. The length of the first antennae in males and the length of the abdominal process in females were measured under 40X magnification using an ocular micrometer. Total body length, defined as the distance from the top of the head capsule to the base of the shell spine, was also measured at these times.

Chemical exposures

The effects of the chemicals diethylstilbestrol (Sigma, St. Louis, MO, USA), methoprene (Chem Service, West Chester, PA, USA), and androstenedione (Sigma, St. Louis, MO, USA) on development of secondary sex characteristics in female and male daphnids was assessed. The vertebrate estrogen agonist diethylstilbestrol was selected because we have shown that this compound reduces molt frequency in immature daphnids

[16] and may interact with the 20-hydroxyecdysone or other steroid receptors in daphnids. The vertebrate androgen androstenedione was selected because we have shown that this steroid elicits developmental toxicity to daphnids [17]. Methoprene, an insect growth regulator, was chosen because its mechanism of action is by mimicking juvenile hormones in insects [18]. The role of terpenoid hormones in crustaceans is equivocal; however, several lines of evidence suggest that terpenoids are involved in the regulation of crustacean reproduction [19, 20].

Daphnids (<24 hours old) were exposed to three different concentrations of each chemical in 250 ml beakers containing 200 ml of medium with two beakers per treatment. Each beaker contained twelve to fifteen daphnids of the same sex. Beakers containing males were fed 1.0×10^7 cells of algae and beakers containing females were fed 3.5×10^7 cells of algae twice daily. Medium was renewed on day 3. The highest exposure concentration of each chemical approximated the no observed effect level as determined during acute toxicity tests with females. Test chemicals were delivered in absolute ethanol. Controls contained the highest concentration of ethanol present in any test solution (0.02%). On days three and six, daphnids from one beaker were used to measure sex characteristics and body lengths using the same techniques used in the determination of normal sexual development.

Data Analysis

The normal relationship between sexual characteristic and body length was determined graphically for both sexes. A scatterplot of the square root of the sexual characteristic length versus the overall body length was found to yield a linear relationship.

A linear regression curve was then fit to the transformed data. On days three and six of the chemical exposures, the predicted sexual characteristic length (SCL_P) for each daphnid was calculated using the daphnid's measured body length and the pre-established linear equation. A reference sexual characteristic length (SCL_R) for each day then was determined. The SCL_R consisted of the mean SCL_P for all daphnids of the same sex analyzed on that day. This value represented the expected sex characteristic length for the group of daphnids based upon the mean total length of the daphnids. The normalized sex characteristic length (SCL_N) of each daphnid was then calculated by using the following equation where SCL_M represents the actual measured sexual characteristic length:

$$SCL_N = SCL_M - SCL_P + SCL_R$$

Using this equation, a daphnid with the same SCL_M and SCL_P would have a normalized sexual characteristic length equal to the SCL_R . Differences between the SCL_M and the SCL_P are reflected in a commensurate increase or decrease in the SCL_R . This normalization corrects for changes in sex characteristic length that are related to effects of the chemical on the overall growth of the organism. Observed body lengths and normalized sexual characteristic lengths were evaluated for significant differences by ANOVA and Dunnett's t-test at an alpha level of 0.05 using JMP software (SAS Institute, Cary, NC).

RESULTS

Sexual Differentiation

Sexual differentiation of the external anatomy of daphnids occurred largely during reproductive maturation. These characteristics could thus be viewed as secondary sex characteristics. Sex of neonatal daphnids could be best discerned by the longer first antennae of males, which rapidly increased in size relative to the size of the daphnid during maturation (between the first and fourth postnatal molts (Fig. 1). The carapace of immature males resembled that of females; however, at the latter stages of reproductive maturation (~ molt 4) the carapace of males underwent a morphological transformation (Fig. 2). At this time, the rostrum of the head capsule was largely lost. The anterior carapace edge below the head capsule developed a protrusion that formed a circular opening rimmed with setae. A prominent characteristic of reproductive maturation in females was the development of the brood chamber and associated abdominal process (Fig. 2). The abdominal process forms the movable posterior boundary of the brood chamber. The abdominal process was not present at release from the mother but rapidly developed in females relative to the increase in total body length between the third and fifth molts (Fig. 1).

The first antenna of males and the abdominal process of females were selected as representative secondary sex characteristics as these were distinctive to each sex, could be easily and objectively measured, and developed at measurable rates during maturation. A linear relationship was discerned between the lengths of these sex characteristics and the body length following appropriate data transformations (Fig. 3). These relationships were

used to determine whether the size of a sex characteristic deviated from the normal size relative to the length of the organism following chemical exposure.

Chemical Exposure

Experiments were next undertaken to determine whether chemical exposure affected the rate of development of the secondary sex characteristics in female and male daphnids. Exposure of female daphnids to 3.0 μM DES significantly reduced the total body length at both days 3 and 6 (Fig. 4). Despite this overall decrease in size, exposure to 3.0 μM DES significantly increased the length of the abdominal process relative to body size. These observations indicate that either: a) DES adversely affected daphnid growth without affecting the development of the abdominal process, or b) DES had a direct stimulatory effect on the growth of the abdominal process. DES had no effect on body size of male daphnids or the development of the first antennae (data not shown).

Methoprene elicited effects on growth and maturation of female daphnids similar to that observed with DES, but with significantly greater potency. Total length of both male and female daphnids was significantly reduced from exposure to 0.16 and 0.32 μM methoprene (Fig. 5). At day 3, this decrease in growth was associated with a significant increase in normalized abdominal process length. At day 6, normalized abdominal process length among daphnids exposed to 0.16 and 0.32 μM methoprene was comparable to controls, however, the normalized abdominal process lengths among daphnids exposed to 0.08 μM methoprene was significantly greater than controls. Like DES, methoprene had no

effect on the normalized first antennae length of male daphnids, although this compound reduced total length of the daphnids (Fig. 6)

Effects of androstenedione on growth and sexual maturation of daphnids were quite distinct from that of DES and methoprene. Exposure of female daphnids to 12 and 25 μM androstenedione significantly reduced total length of female daphnids, but had no effect on normalized abdominal process length (Fig. 7). Male daphnids were more sensitive to the effects of androstenedione. All males exposed to 25 μM androstenedione died by day 6. Exposure to 6.2 and 12 μM androstenedione for 6 days had no effect on total length of male daphnids, but significantly increased normalized length of the first antennae of daphnids (Fig. 8). These results demonstrate that androstenedione had a direct impact on the development of first antennae of males that was independent of any effects on overall growth of the daphnids.

DISCUSSION

Little is known of the processes that govern sexual differentiation in lower crustaceans such as cladocerans. In decapod crustaceans (i.e. crabs, lobsters, shrimp) and other malacostracans, a loosely arranged aggregate of cells associated with the testis called the androgenic gland directs male differentiation [21-23]. In the absence of the androgenic gland, these crustaceans develop the female phenotype. Several studies report the masculinization of female crustaceans after implantation of androgenic glands from males [24,25]. Likewise, ablation of the androgenic gland in males leads to demasculinization [26-28]. In isopods the substance secreted by this gland, the Androgenic Hormone, has been shown to be a protein [29]. The androgenic gland has not yet been described in cladocerans; however, a comparable organ or cell type may be responsible for masculinization in this species. Nevertheless, precedence supports the involvement of the endocrine system in sexual differentiation in crustaceans and these processes may therefore be targeted by endocrine disrupting chemicals.

Several sex-specific characteristics were identified in the present study that developed during the reproductive maturation of daphnids. Daphnids could be monitored for the effects of chemical exposure on the time required for the development of these characteristics; however, their rapid appearance precludes assessments of the effects of chemical exposure on their rate of development. In contrast, the lengths of the first antennae of males and the abdominal process of females increase at rates that can be accurately measured over several molt periods. For these reasons, first antennae growth and abdominal process growth in

males and females, respectively, were selected to monitor chemical effects on the development of secondary sex characteristics of daphnids.

Toxicants may affect development of secondary sex characteristics as a consequence of effects on the overall growth of daphnids. Such effects would reflect an overall growth effect rather than a specific effect on the development of the secondary sex characteristics. The normal relationships between the development of the secondary sex characteristics of male and female daphnids and overall body length therefore were determined in order to correct for overall effects of chemical exposure on growth of the organisms. Deviations from these relationships resulting from chemical exposure would indicate a specific effect on the development of the respective sexual characteristic. Using these established relationships the effects of chemical exposure on the development of the secondary sex characteristics were then determined.

DES and methoprene elicited remarkably similar effects on the development of secondary sex characteristics of daphnids. Both compounds reduced overall growth while increasing normalized abdominal process length in females. Both compounds had no effect on the development of first antennae in males. DES [16] and methoprene [30] have both been shown to reduce molt frequency in female daphnids. This common effect of both compounds is likely responsible for the reduced growth of female daphnids observed in the present study. However with both chemicals, this reduction in growth was not accompanied by a commensurate decrease in length of the abdominal process, as was observed during the exposure of female daphnids to 25 μM androstenedione. These observations suggest that

either: a) growth and development of the abdominal process are independently regulated and these compounds specifically affect daphnid growth, or b) these compounds stimulated the rate of development of the abdominal process resulting in abdominal processes that were larger than normal for the size of the organisms.

Baldwin et al. [16] hypothesized that DES interferes with daphnid molting by binding antagonistically to the ecdysone receptor. In insects, juvenile hormone has been shown to bind to ultraspiracle, a protein that forms heterodimers with the ecdysone receptor, and in doing so modulates the action of ecdysteroids [31, 32]. Methyl farnesoate may have a function in crustaceans similar to that of juvenile hormone in insects [19, 20]. Methyl farnesoate appears to function as a gonadotropin in crustaceans [33] and has been shown to stimulate ovarian maturation in crustaceans [33, 34] as well as induce larval metamorphosis in the barnacle [35]. Methoprene may bind to a putative methyl farnesoate receptor in an antagonistic manner. Interactions between the daphnid ecdysone receptor and the methyl farnesoate receptor, as observed with the ecdysone receptor and ultraspiracle in insects, thus may provide a common mechanism by which DES and methoprene elicit similar effects on daphnids.

The steroidal compound androstenedione has been shown to cause developmental abnormalities in daphnids [17]. Androstenedione is the precursor to testosterone and we have demonstrated that daphnids possess significant 17β -hydroxysteroid dehydrogenase activity, the activity responsible for the conversion of androstenedione to testosterone [36, 37]. We also have demonstrated that androstenedione [17] and testosterone cause

developmental abnormalities in daphnids, as does exposure to compounds that alter testosterone metabolism in a manner that would result in elevated testosterone levels [17]. These observations suggest that steroidal androgens have a specific target site of toxicity. A functional role of steroidal androgens has not been firmly established though testosterone has been reported to have androgen-like activity in some crustaceans [38, 39]. In the present study, androstenedione was shown to be more toxic to male as compared to female daphnids. The highest level of androstenedione assessed was specifically lethal to male daphnids, and lower levels specifically stimulated normalized first antennae development. These observations demonstrate both the potential for differential susceptibility of female and male daphnids to the toxicity of chemicals and the susceptibility of the development of male secondary sex characteristics to chemical exposure.

In conclusion, results from this study demonstrate that the development of secondary sex characteristics of daphnids can be readily monitored during chemical toxicity assessments. The developments of these characteristics are susceptible to the effects of some chemicals and the detection of such effects may prove to be indicators of endocrine-related toxicity to crustaceans. Results also revealed that male daphnids are more susceptible to the toxicity of some chemicals, as demonstrated with androstenedione, and standard toxicity assessments using only parthenogenetically-reproducing females may underestimate the toxicity of such chemicals to daphnid populations. These observations are consistent with observed sex differences in susceptibility to chemical toxicity in other species [40] and highlight the need to modify existing toxicity testing protocols with daphnids to include both sexes.

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FIGURE LEGENDS

Figure 1. Maturation of Female and Male Daphnids. Female body size, abdominal process size, and the ratio of abdominal process:body lengths versus age for female daphnids. Male body size, first Antenna size, and the ratio of first antenna:body lengths versus age for male daphnids. Data points (mean \pm standard deviation) was derived from 6 female or 12 male daphnids.

Figure 2. Secondary sex characteristics in adult daphnids. A. Female. An elongated rostrum is present on the head capsule (1). Carapace edge below the head capsule is smooth with no setae present (2). First antennae are diminutive (3). A long abdominal process forms the posterior boundary of the brood chamber (4). B. Male. Frontal portion of the head capsule is flattened (1). Carapace edge below the head capsule is indented and lined with setae (2). Primary antennae are elongated (3). Abdominal process is absent (4).

Figure 3. Relationship between sexual characteristics and body lengths. A. Female – Square root of the abdominal process length versus total body length. B. Male – Square root of the first antenna length versus total body length.

Figure 4. Effect of diethylstilbesterol on total body and normalized abdominal process length of female daphnids. Data are presented as the mean and standard deviation (n=10-13). Normalization of the secondary sex characteristics to body length is described in the materials and methods section. An asterisk indicates a significant (≤ 0.05) difference from the control (Dunnett's t-test).

Figure 5. Effect of methoprene on total body and normalized abdominal process length of female daphnids. Data are presented as the mean and standard deviation (n=14-16). Normalization of the secondary sex characteristics to body length is described in the materials and methods section. An asterisk indicates a significant (≤ 0.05) difference from the control (Dunnett's t-test).

Figure 6. Effect of methoprene on total body and normalized first antennae length of male daphnids. Data are presented as the mean and standard deviation (n=12-15). Normalization of the secondary sex characteristics to body length is described in the materials and methods section. An asterisk indicates a significant (≤ 0.05) difference from the control (Dunnett's t-test).

Figure 7. Effect of androstenedione on total body and normalized abdominal process length of female daphnids. Data are presented as the mean and standard deviation (n=10-13). Normalization of the secondary sex characteristics to body length is described in the materials and methods section. An asterisk indicates a significant (≤ 0.05) difference from the control (Dunnett's t-test).

Figure 8. Effect of androstenedione on total body and normalized first antennae length of male daphnids. Data are presented as the mean and standard deviation (n=7-12). Normalization of the secondary sex characteristics to body length is described in the

materials and methods section. An asterisk indicates a significant (≤ 0.05) difference from the control (Dunnett's t-test).

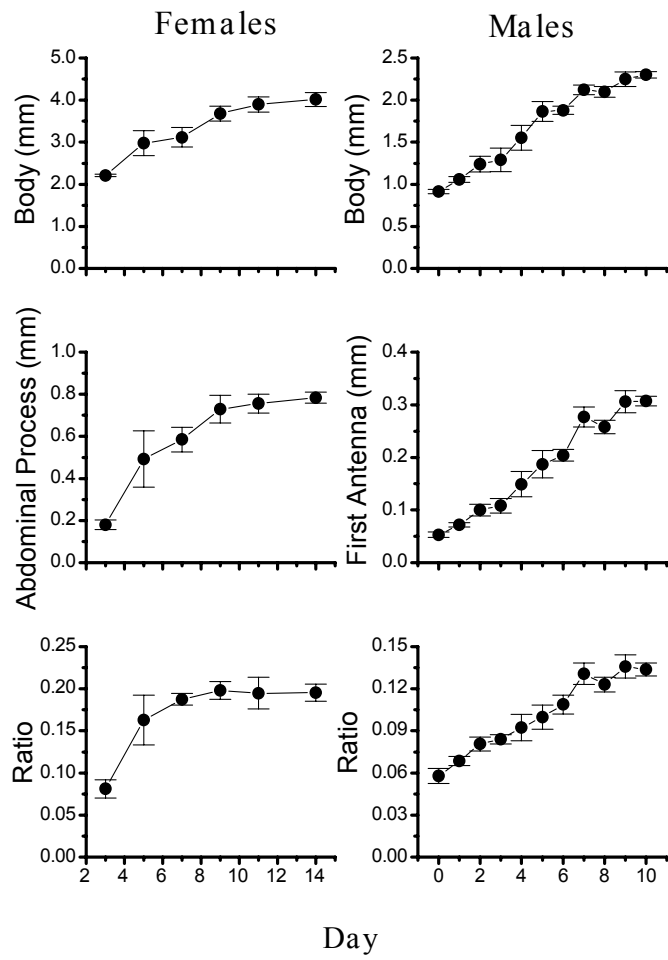


Figure 1

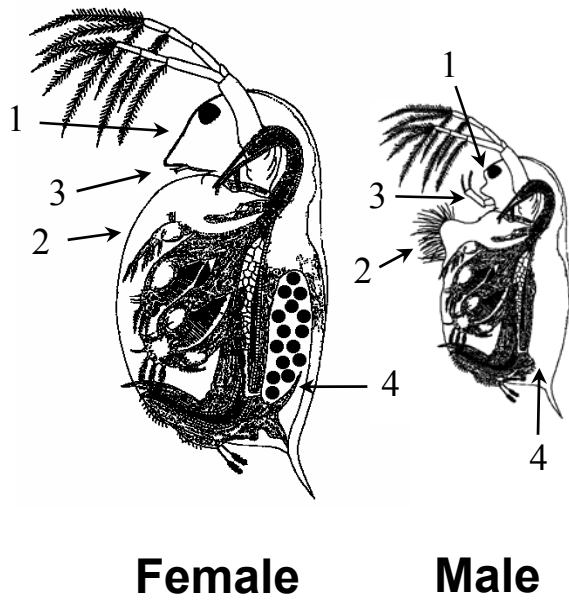


Figure 2

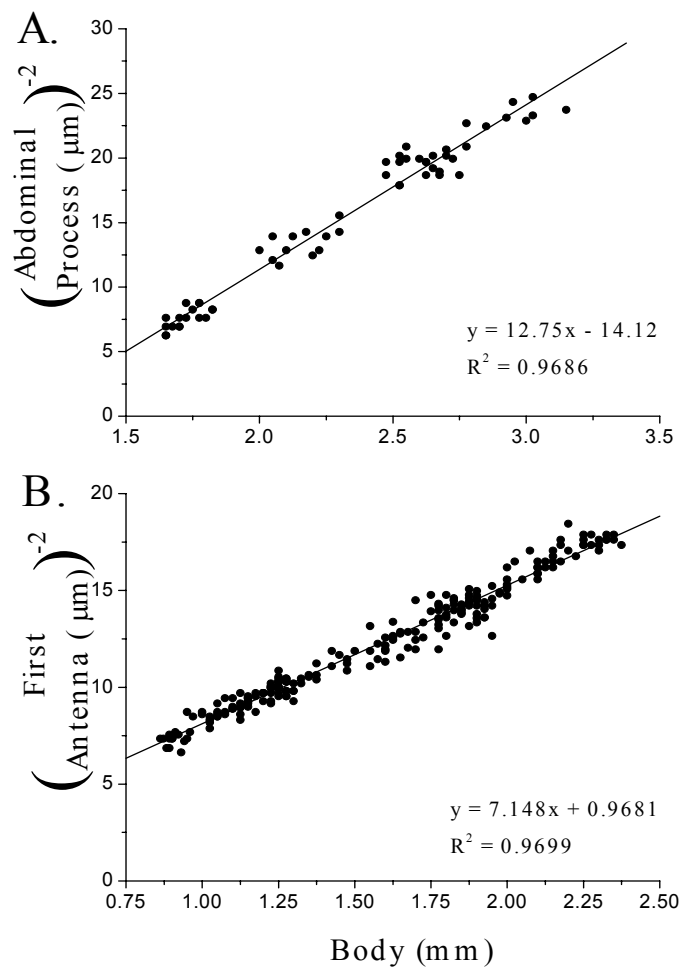


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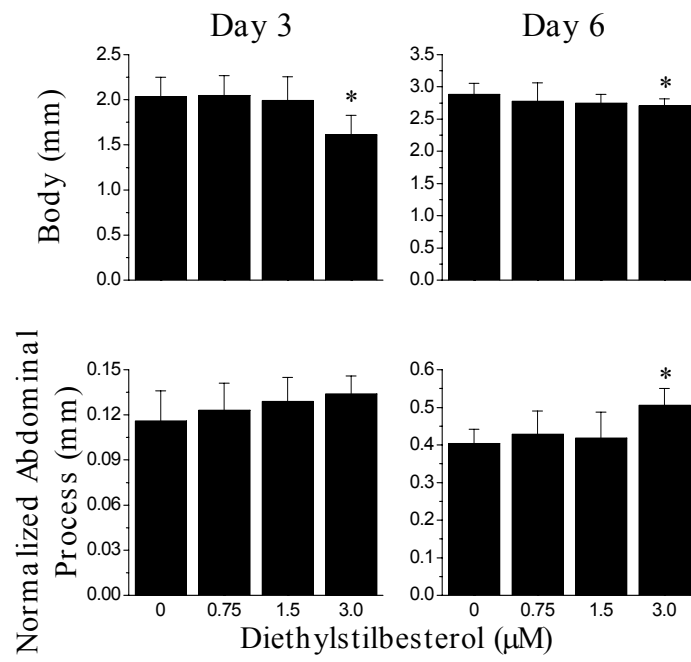


Figure 4

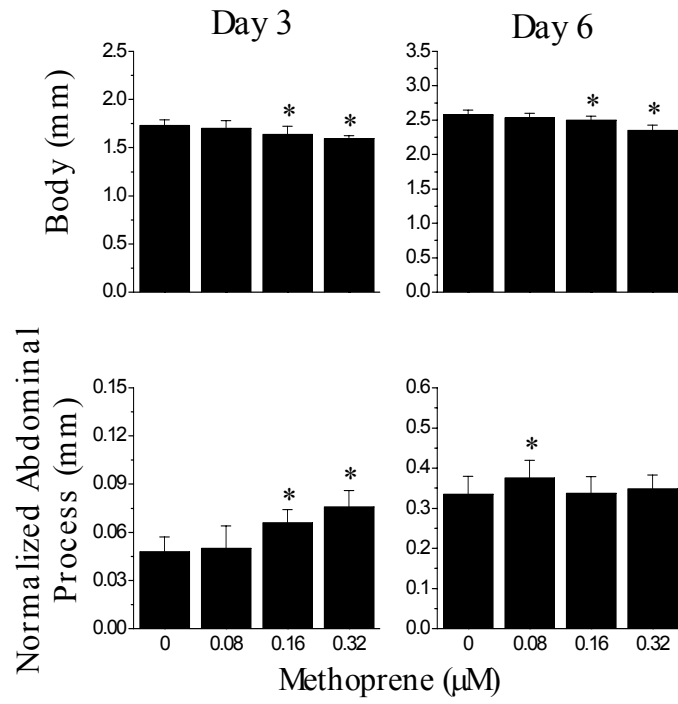


Figure 5

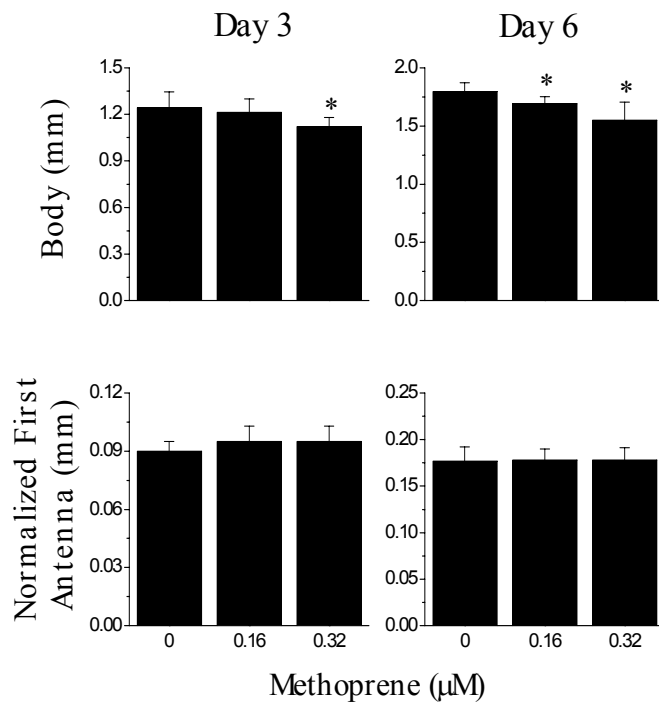


Figure 6

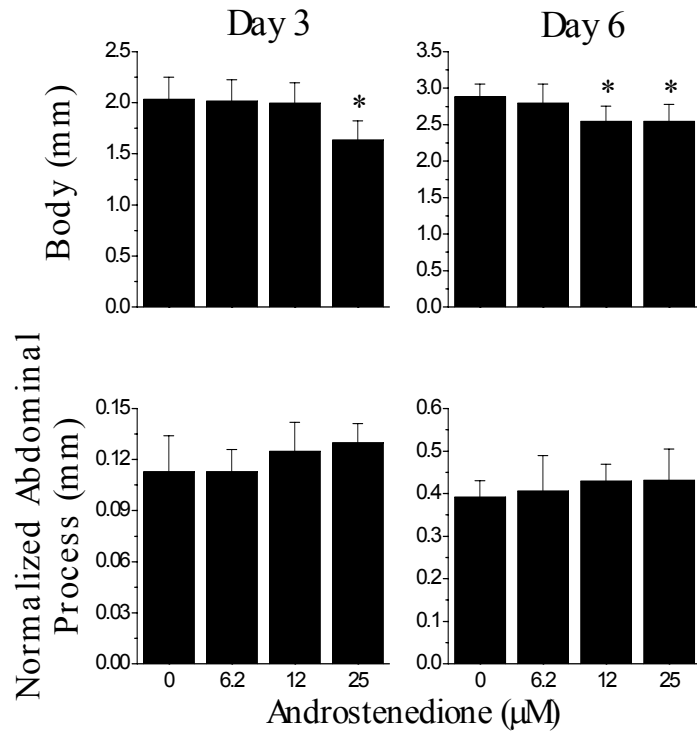


Figure 7

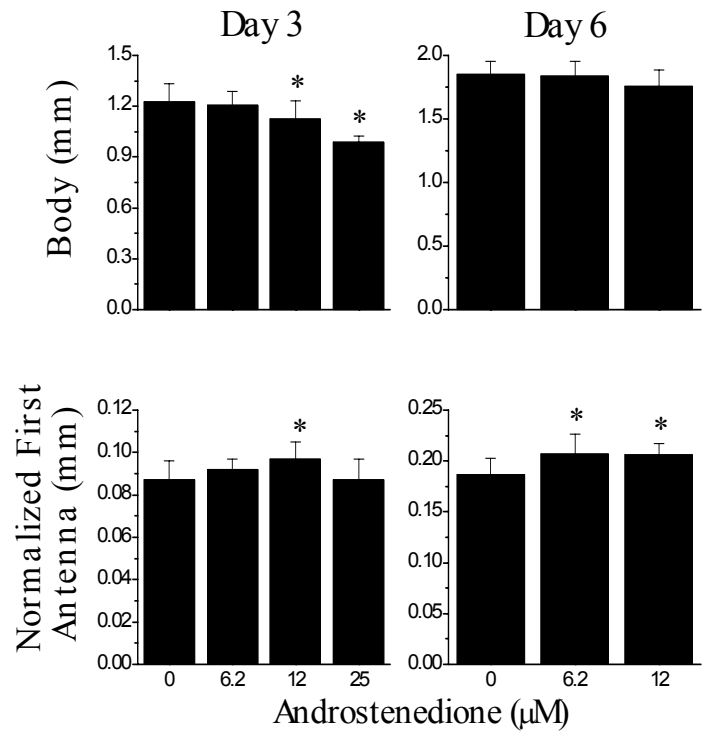


Figure 8

**Low Exposure Concentration Effects of Methoprene on
Endocrine-Regulated Processes in the Crustacean
*Daphnia magna***

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and is in the format required of that journal

ABSTRACT

Methoprene is a growth-regulating insecticide that manifests its toxicity to target organisms by acting as a juvenile hormone agonist. Methoprene similarly may exert toxicity to crustaceans by mimicking or interfering with methyl farnesoate, a crustacean juvenoid. We hypothesized that methoprene interferes with endocrine-regulated processes in crustaceans by several mechanisms involving agonism or antagonism of juvenoid receptor complexes. In the present study, we evaluated this hypothesis, in part, by characterizing and comparing the concentration-response curves for methoprene and several endpoints related to development and reproduction of the crustacean *Daphnia magna*. Our results demonstrate that methoprene has multiple mechanisms of toxicity and low exposure concentration effects. Methoprene reduced the growth rate of daphnids with evidence of only a single concentration-response line having a threshold of 12.6 nM. Molt frequency was reduced by methoprene in a concentration-dependent manner with a response curve corresponding to a two-segmented line with thresholds at 4.2 and 0.21 nM. An endpoint related to reproductive maturation, the time of first brood deposition, also was affected by methoprene, with a clear concentration-dependent response and a NOEC of 32 nM. Methoprene reduced fecundity according to a two-segmented line with thresholds of 24 and ≤ 0.18 nM. These results demonstrate that methoprene elicits significant toxicity to endocrine-related processes in the 5-50 nM concentration range. Furthermore, molting and reproduction were impacted at significantly lower methoprene concentrations with a distinct concentration-response and a threshold of ≤ 0.2 nM. The different concentration-dependent response to methoprene could involve agonism or antagonism of various juvenoid receptor configurations.

INTRODUCTION

Insect growth regulators are insecticides that are designed to disrupt specific physiological processes of target insects. These insecticides typically perturb enzymatic and hormonally regulated processes that are relatively specific to insect physiology (Retnakaran et al., 1985; Dhadialla et al., 1998). These designer pesticides usually have low toxicity to non-target organisms because these species generally lack the enzymatic or hormonally-regulated processes that these insecticides disrupt. Methoprene is one of the most widely used and successful insect growth regulators and elicits toxicity to target insects by acting as a juvenile hormone agonist (Retnakaran et al., 1985). One of the main uses of methoprene is in the control of mosquitoes, where it prevents the metamorphosis of mosquito larva into adults, and in this capacity, is applied directly to the aquatic environment often as pelleted, sustained-release or liquid formulations (Retnakaran et al., 1985; Dhadialla et al., 1998).

Juvenile hormone (JH) is a sesquiterpenoid that bears structural similarity to the terpene component of retinoic acid. JH modulates ecdysteroid activity in insects and also influences reproduction, caste determination, behavior, diapause, and metabolism (Nijhout, 1998). In vertebrates, retinoids are ligands to retinoic acid receptors (RAR) and retinoid X receptors (RXR) (Mangelsdorf et al., 1990). Liganded RXR can homodimerize or can heterodimerize to RAR, some steroid receptors and other receptors to form transcriptional activators (Harmon et al., 1995; Mangelsdorf and Evans, 1995). USP is an insect homolog of the RXR (Yao et al., 1992), which heterodimerizes to the ecdysone receptor (EcR) to form the ecdysone transcriptional activator (Yao et al., 1993). USP also can heterodimerize with partner receptors to RXR (Yao et al., 1992) demonstrating its functional homology to RXR.

Like RXR, it is possible that USP also stimulates transcriptional activation by homodimerization. The endogenous ligand to USP has not been unequivocally established; however, USP was shown to bind juvenile hormones *in vitro* and the complex was shown to induce USP-dependent transcription of a reporter gene assay (Jones and Sharp, 1997). JH analogs also have been shown to bind and activate RXR in mammals (Harmon et al., 1995). Taken together, these observations indicate that 1) USP functions as an insect homolog to RXR, 2) JH is the ligand to USP, and 3) USP mediates the regulatory influence of JH either by heterodimeric combination with other receptors such as EcR or through homodimerization.

Crustaceans apparently do not utilize juvenile hormones as do insects (Chang, 1993). However, the related juvenoid, methyl farnesoate, the unepoxidated form of Juvenile Hormone III, may be an important reproductive hormone in crustaceans (Laufer et al., 1993; Homola and Chang, 1997; Lu et al., 2000). Methyl farnesoate has been shown to stimulate ovarian maturation in crustaceans (Laufer et al., 1998; Reddy and Ramamurthi, 1998), induce larval metamorphosis in the barnacle (Yamamoto et al., 1997), and increase molt duration in the larvae of lobster (Borst et al., 1987) and shrimp (Abdu et al., 1998).

In light of the structural and functional homology between insect juvenile hormones and crustacean methyl farnesoate, we hypothesized that the juvenile hormone analog methoprene may specifically target processes in crustaceans that are regulated by methyl farnesoate. Methoprene has been reported to reduce fecundity of mysid shrimp (McKenney and Celestial, 1996) and cladocerans (Templeton and Laufer, 1983; Chu et al., 1997) and

interfere with normal juvenile development of grass shrimp (McKenney and Matthews, 1990), mud crab (Celestial and McKenney, 1994), and daphnids (Templeton and Laufer, 1983). These effects have commonly been reported to occur in the 30 to 300 nM exposure concentration range. In contrast, EC50 values for emergence of mosquitoes have been reported in the aqueous concentration range of 0.03 to 3 nM (Schaefer and Wilder, 1973; Ritchie et al., 1997). These observations would suggest that methoprene is a significantly less potent analog of methyl farnesoate as compared to juvenile hormone and crustaceans are less susceptible to the endocrine-disrupting toxicity of methoprene as compared to insects.

We have hypothesized that the toxicity of methoprene to crustaceans is mediated by its interaction with one or more RXR/USP family members resulting in agonism or antagonism of receptor dimers that utilize RXR/USP. Some of these interactions may result in toxicity at exposure concentrations rivaling those that affect juvenile hormone-regulated processes in target insects. Modeling of concentration-responses of the effects of methoprene on several endocrine-regulated processes in the crustacean, *Daphnia magna*, was performed using a two-segmented linear approach in an effort to discern multiple mechanisms of methoprene toxicity and establish threshold concentrations for the observed effects.

MATERIALS AND METHODS

Daphnid Cultures

Daphnids were cultured in medium as described previously (Baldwin and LeBlanc, 1994). Daphnids were fed green algae (*Selenastrum capricornutum*) supplemented with Tetrafin fish food (Pet International, Chester Hill, New South Wales, Australia). Algae were cultured in Bold's basal medium (Nichols, 1973). The fish food supplement was prepared by homogenizing 10 g of fish food in a blender with 1 L of water for 10 minutes. Solids were settled out overnight and the resulting supernatant was used. Aliquots of the supernatant were dried at 100°C to determine the suspended solids content. Daphnid cultures (1 L medium containing 40 daphnids) were fed 1.4×10^8 cells of algae twice daily and 4 mg (dry weight) of fish food suspension twice daily. Daphnids were transferred to new medium and offspring removed three times weekly. Cultures and all experiments were maintained at 20°C at a 16 hour photoperiod.

Influence of Methoprene on Growth, Maturation, and Reproduction

All experiments were conducted at methoprene exposure levels of <300 nM. Prior studies conducted in our laboratory have indicated that daphnids would tolerate these exposure levels without signs of overt toxicity (Olmstead and LeBlanc, 2000). Definitive concentration-response analyses of the effects of methoprene on growth, molting, attainment of reproductive maturity, and fecundity were evaluated using one of two experimental designs.

Experimental Design #1: Daphnids (<5 hours old) were exposed to 70 concentrations of methoprene (Chem Service, West Chester, PA, USA). One daphnid was exposed to each concentration of methoprene and each methoprene concentration was 90% of the next greater concentration. Ten daphnids (controls) were also individually exposed to the same amount of carrier solvent (0.005% absolute ethanol) as was present in all methoprene treatments. One daphnid was exposed to each treatment level in a 50 mL beaker containing 40 mL of test solution. Algae (3.5×10^6 cells) and fish food supplement (100 μg dry weight) was provided to each beaker twice daily during the first week of each experiment. Subsequently, 7.0×10^6 cells of algae and 200 μg fish food supplement (dry weight) were provided to each beaker twice daily. Daphnids were transferred to fresh media three times weekly. Offspring were counted and removed from the beakers daily. The impact of methoprene exposure on offspring production was assessed for each brood through the first five broods. Solutions were examined hourly for the presence of molted exoskeletons. Growth rates of daphnids were established by measuring molted exoskeletons from the base of the shell spine to the top of the carapace. Growth rates of daphnids are linear over the first week of life (Olmstead and LeBlanc, 2000), therefore, growth rates were determined as the slope of the linear fit of the graph of molt size versus molt number for the first 5 molts (~6 days). This design provided for definitive characterization of the methoprene concentration-response lines.

Experimental Design #2: Daphnids (<5 hours old) were exposed to 6 different concentrations of methoprene or carrier solvent (0.025% absolute ethanol) with 10 daphnids individually exposed to each treatment. The experimental design was otherwise the same as described for experimental design #1. Time to reproductive maturation was assessed using this design.

Time to reproductive maturation was denoted as the time at which the first clutch of eggs were transferred from the ovaries to the brood chamber. Transparency of the daphnids allowed for visual determination of this endpoint without having to manipulate the organisms in any way. This endpoint represents the end of the reproductive maturational period which is the instar at which oocyte development begins in the ovaries. This design was necessary to assess the effects of methoprene on the time required for daphnids to attain reproductive maturation since high methoprene concentrations were found to delay the transfer of eggs from the ovaries to the brood chamber by one molt period (6th versus 5th instar). As a result, concentration-response lines generated using experimental design #1 did not represent a continuous concentration-response, but rather, resulted in a best-fit between daphnids that attained reproductive maturity during the fifth instar and those that attained reproductive maturity during the sixth instar.

Data Analysis

Concentration response curves were fitted with a two-segmented linear model using the PROC NLIN, MARQUARDT method using SAS 7.0 software (SAS Institute, Cary, NC). The following model was fit to the data:

$$x \leq t : y = b + m_1 * x$$

$$x > t : y = b + m_1 * x + m_2 * (x - t)$$

Where y is the measured variable, x is the logarithm of the methoprene concentration, t is the logarithm of the methoprene concentration at which the two linear segments intersect, and m₁ and m₂ are the slopes of the lower and higher concentration segments, respectively. Each segment of the model fits were then analyzed by linear regression in order to test if the slopes

were significantly different from zero using an F-test (Zar, 1996). Threshold concentrations were defined as the concentration at which the concentration-response line intersected the line representing the appropriate control.

Differences in reproductive maturation time were deemed statistically significant using an analysis of variance and Dunnett's t-test (Zar, 1996) at an alpha level of 0.05 with JMP software (SAS Institute, Cary, NC). Growth rates were calculated as the slope of the linear regression fit of molt length versus molt number for individual daphnids.

RESULTS

Juvenile Molt and Growth Rates

Molting and growth are closely coordinated in arthropods since molting of the exoskeleton must occur to allow for the expansion of body mass associated with growth (Pennak, 1953). Molting is stimulated by 20-hydroxyecdysone in conjunction with the ecdysone transcriptional activator (Chang, 1993). Growth also is likely to be regulated by this or a similar hormone-receptor complex. We therefore hypothesized that methoprene would coordinately interfere with molting and growth.

Methoprene significantly reduced growth rate among juvenile daphnids in a concentration-dependent manner with a highly significant slope ($P < 0.0001$) and a clear threshold evident at 12.6 nM (Fig. 1). Methoprene also significantly reduced juvenile molt frequency, as measured by the duration of the intermolt period (instar) between the first and second molt (Fig. 2). This response conformed to a two-segmented line with each having a significant non-zero slope. The threshold concentration for this response at higher concentrations was 4.2 nM with a highly significant slope ($P < 0.0001$). The second concentration-response line was evident at lower methoprene concentrations with a significant slope ($P = 0.012$). This response exhibited a threshold at 0.21 nM.

Reproductive Maturation

Reproductive maturation of daphnids involves multiple coordinated events that are likely to be influenced by juvenoid hormones. These include growth – the organism must be of adequate size to accommodate the brood of eggs deposited into the brood chamber from

the ovaries; and, the timing of molting – egg maturation in the ovaries, deposition of eggs into the brood chamber, and release as fully developed offspring all must be timed with the occurrence of a molt. The effect of methoprene on the time of first deposition of eggs into the brood chamber was evaluated. Methoprene significantly increased the time to first brood deposition according to a single concentration-response line exhibiting an NOEC of 32 nM (Fig. 3).

Fecundity

Modeling of the concentration-responses for the impact of methoprene on brood sizes yielded a segmented line with two non-zero slopes. At high exposure concentrations (> 24 nM) methoprene reduced fecundity of daphnids in a manner characterized by a steep concentration-response line (Fig. 4) that progressively increased in magnitude with increasing cumulative brood number (Fig. 5). The threshold concentration for this effect was 24 nM after five cumulative broods with a highly significant slope ($P < 0.0001$) (Fig 4).

Methoprene adversely affected fecundity of daphnids at lower exposure concentrations (< 24 nM) with a concentration-response line appreciably more shallow than that observed at higher concentrations (Fig. 4). The slopes of the concentration-response lines at the low methoprene exposure concentrations did not appreciably change with progressing cumulative broods (Fig. 5). The slope for this effect after five cumulative broods was significant ($P = 0.03$) with a threshold of < 0.18 nM, the lowest concentration evaluated.

DISCUSSION

This study was based upon the premise that methoprene can elicit toxicity to crustaceans through various receptor-mediated interactions. Concentration-response lines were generated for several endocrine-regulated processes using a two-segmented linear model in an attempt to discern differences in the concentration-responses that would be indicative of different mechanisms of toxicity and perhaps reveal effects of methoprene at low exposure levels. Results provided evidence for both multiple mechanisms of methoprene toxicity to crustaceans and threshold concentrations for some of these effects that were significantly lower than previously reported for crustaceans.

All of the physiological endpoints measured were negatively impacted by methoprene at concentrations greater than 30 nM. These effects are consistent with those typically reported for methoprene and crustaceans (Templeton and Laufer, 1983; McKenney and Matthews, 1990; Celestial and McKenney, 1994; McKenney and Celestial, 1996; Chu et al., 1997). The generalized adverse response of all of the measured physiological endpoints to these exposure concentrations suggests that methoprene impacted processes that were critical to the general fitness of the organisms. For example, the RXR family of receptors is involved in regulating several aspects of lipid metabolism and utilization in vertebrates (Repa, 2000). Methoprene has been shown to activate gene transcription from an RXR response element (Harmon et al., 1995). At the higher exposure concentrations used in this study, perhaps methoprene interacted with an RXR/USP family member in a manner that resulted in aberrant lipid metabolism. Limitations in fuel (lipids) for energy may have resulted in uniform adverse effects on the measured growth and reproductive endpoints.

The concentration-responses generated with molting and fecundity conformed to a two-segmented line with each segment having a non-zero slope. The common concentration-response of these endpoints at the low methoprene concentrations suggests that these two endpoints share a common target of methoprene toxicity that is different from that responsible for the high-concentration effects. The molting process in arthropods is initiated by a drop in ecdysteroid levels and the receptor heterodimer EcR-USP mediates this response (Yao et al., 1993). EcR-USP also is known to regulate oogenesis in insects (Carney and Bender, 2000). Thus, a high affinity interaction of methoprene with USP/RXR (Harmon et al., 1995) may have modulated EcR-USP activity resulting in the observed low-concentration effects of this compound on molting and reproduction.

Reproductive maturation denotes the end of the juvenile phase and the beginning of the adult phase in daphnids. Energy resources previously allocated to growth, during the juvenile stage, are largely directed towards reproduction during the adult stage. Among daphnids, reproductive maturation commences with the maturation of the ovaries and the development of diploid eggs that develop via parthenogenesis (Pennak, 1953). These eggs are deposited into the brood chamber following the first adult molt. Reproductive maturation therefore begins one instar before that at which eggs are deposited into the brood chamber. Methyl farnesoate has been shown to stimulate ovarian growth and maturation in crustaceans (Laufer et al., 1992; Laufer et al., 1998). Exposure to the higher concentrations of methoprene in the present study delayed reproductive maturation. This endpoint was not amenable to definitive concentration-response analyses as were the other endpoints. However, results suggest that methoprene may have affected this endpoint through the

competitive inhibition of methyl farnesoate or in a manner similar to the high-concentration effects observed with the other endpoints (i.e., altered lipid metabolism).

The definitive concentration-response analyses performed in the present study identified responses of daphnids that were unique to molting and fecundity that exhibited thresholds <0.2 nM. These observations demonstrate that crustaceans do respond to methoprene at concentrations known to affect aquatic insects (Schaefer and Wilder, 1973; Ritchie et al., 1997). The use of methoprene at recommended application rates would be expected to result in environmental concentrations of ~30 nM (Ingersoll et al., 1999). Methoprene concentrations in natural and experimentally enclosed surface waters following application at recommended rates have typically ranged from 3.0 – 30 nM (Knuth, 1989; Ross et al., 1994). Results from the present study indicate that methoprene adversely impacts some endocrine regulated processes in daphnids at concentrations significantly below environmental concentrations at application. Establishing whether the effects elicited at these environmentally-relevant concentrations are sufficient to adversely impact crustacean populations awaits additional study.

ACKNOWLEDGEMENT

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FIGURE LEGENDS

Figure 1. Growth rate of daphnids exposed to concentration of methoprene. Each point represents a single daphnid. Growth rate was calculated as the slope of the linear regression line of a plot of molt length versus molt number for each individual daphnid. The dashed and dotted lines represent mean and standard deviation, respectively, of the growth rate associated with the control daphnids. Solid lines represent the two-segmented line model fit of the data.

Figure 2. Molt frequency of daphnids exposed to concentrations of methoprene as measured by the duration of the second intermolt period (instar). Each point represents a single daphnid. The dashed and dotted lines represent mean and standard deviation, respectively, of the duration of second instar associated with the control daphnids. The solid lines represent the two-segmented line model fit of the data.

Figure 3. Age at which daphnids exposed to concentration of methoprene attained reproductive maturation as measured by the initial transfer of eggs to the brood chamber. Data are presented as the mean and standard deviation (n=10). An asterisk indicates a significant (≤ 0.05) difference from the control (ANOVA, Dunnett's t-test).

Figure 4. Concentration response lines for cumulative offspring production by daphnids exposed to concentrations of methoprene. Each point represents a single daphnid. The dashed and dotted lines represent mean and standard deviation, respectively,

of offspring production associated with the control daphnids. Solid lines represent the two-segmented line model fit of the data.

Figure 5. Two-segmented line model slopes of the concentration response curves for cumulative offspring production of daphnids exposed to concentrations of methoprene.

The slopes of the models in Figure 1 were plotted against the cumulative brood number that the two-segmented line models were fit to. Squares represent the slopes of the lines generated with methoprene concentrations <24 nM and circles represent the slopes of the lines generated with methoprene concentrations >24 nM. Brackets represent the 95% confidence interval associated with each slope.

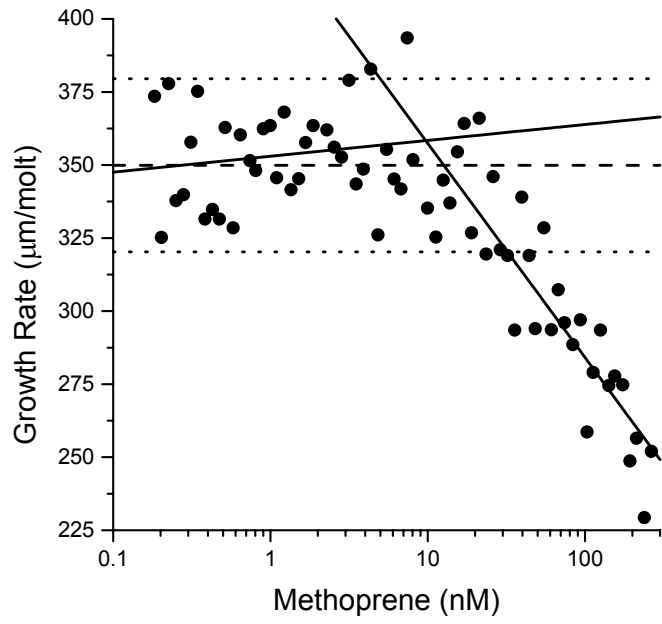


Figure 1

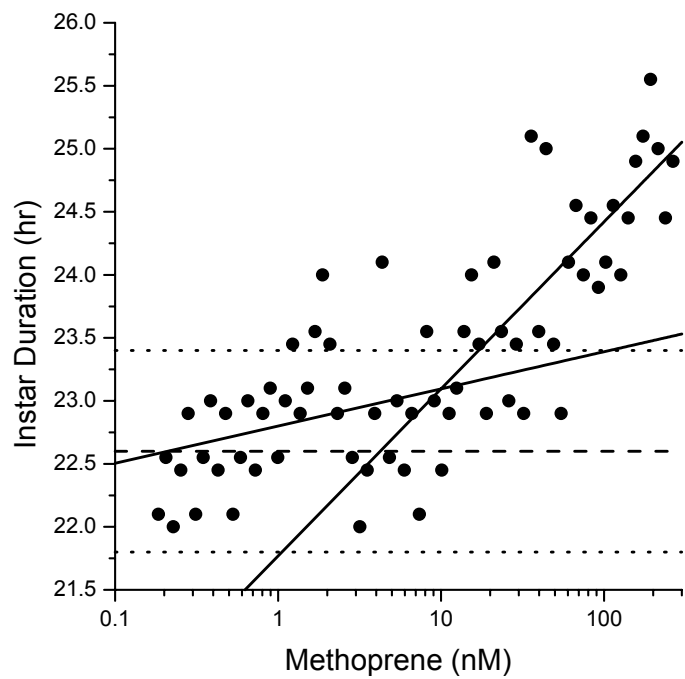


Figure 2

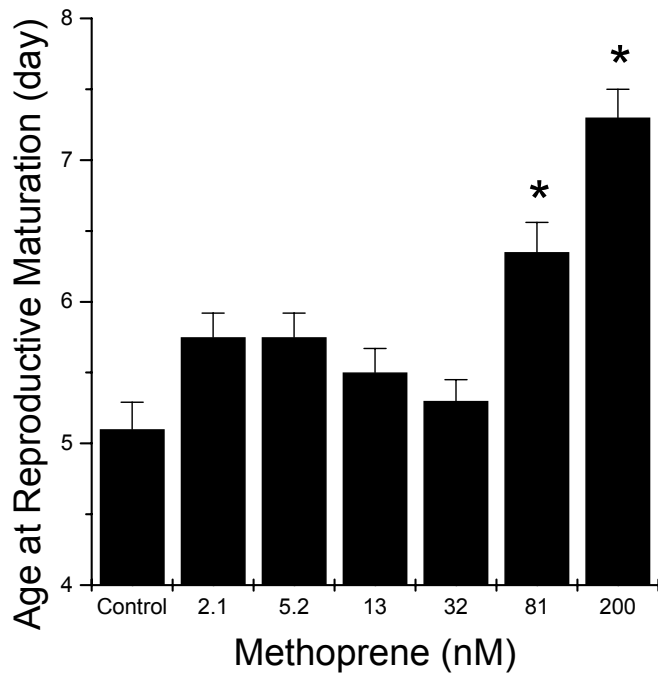


Figure 3

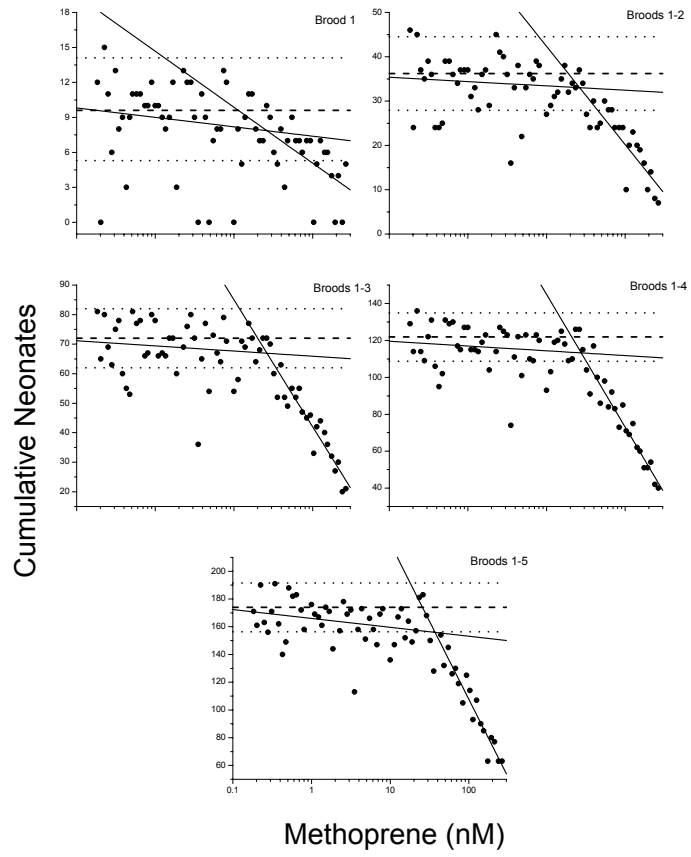
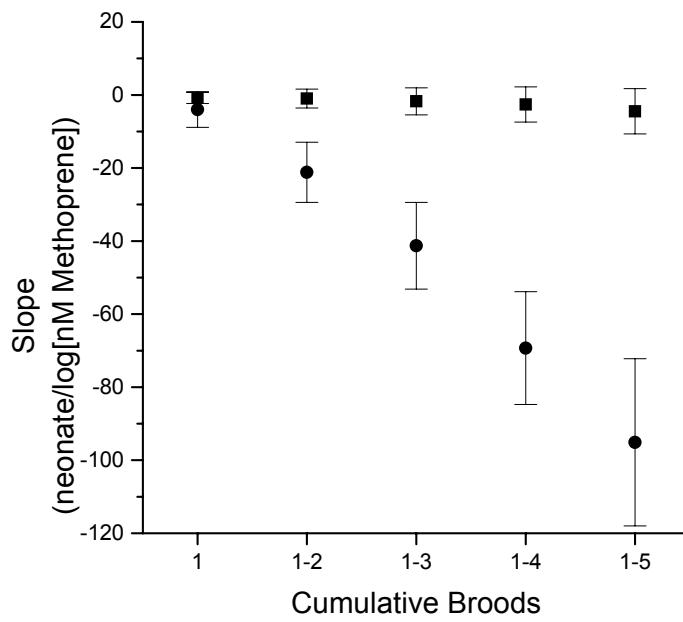


Figure 4

Figure 5



**Temporal and Quantitative Changes in Sexual
Reproductive Cycling of the Cladoceran *Daphnia magna*
by a Juvenile Hormone Analog**

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ABSTRACT

Cyclic parthenogens, such as the cladoceran, *Daphnia magna*, utilize both asexual (parthenogenetic) and sexual reproduction in order to maximize population fitness in variable environments. Parthenogenetic reproduction is the default strategy among *D. magna*, while various environmental cues trigger cycles of sexual reproduction. Experiments were conducted with the juvenile hormone analog methoprene to test the hypothesis that members of the insect juvenile hormone/vertebrate retinoic acid family of transcription factors are involved in the regulation of sexual reproduction in daphnids. Neither methoprene, food reduction, or crowding independently stimulated entry into the sexual reproductive phase of the daphnids. However, the combination of food deprivation and crowding stimulated entry into the sexual reproductive phase characterized by an initial high production of males and the subsequent intermittent production of haploid egg-containing ephippia. Exposure to 160 nM methoprene along with food deprivation and crowding caused a significant reduction in the percentage of males produced during the early phase of the sexual cycle and significantly increased the percentage of males produced during the later stages of the cycle. Methoprene concentrations as low as 6.4 nM significantly reduced the number of resting eggs produced and proportionately increased the production of parthenogenetically-produced neonates. These experiments demonstrate that methoprene uncouples the coordinated production of males and resting eggs during the sexual reproductive period of *D. magna*. Methoprene stimulates male offspring production and defers their production to latter stages of the sexual reproductive period, while inhibiting the production of resting eggs and promoting the continuance of parthenogenetic reproduction.

INTRODUCTION

Species that reproduce by cyclic parthenogenesis are able to utilize both sexual and asexual reproductive strategies during their life histories. The water flea, *Daphnia magna* (Cladocera, Crustacea), is an example of one such species. Asexual (parthenogenetic) reproduction provides for rapid expansion of daphnid populations during times of high resource availability (Hebert, 1978; Lynch and Gabriel, 1983). When resources are depleted, or environmental conditions threaten survival of a population, daphnids switch to sexual reproduction. Sexual reproduction results in genetic exchange and the production of dormant 'resting eggs' that can survive prolonged periods of environmental adversity (Grebelyni, 1996; Hebert, 1978). These resting eggs are encased in ephippia which are highly pigmented casings that protect the eggs. Resting eggs also are amenable to transport to other locals through atmospheric currents or animal attachment (Mellors, 1975; Pennak, 1953).

During favorable environmental conditions a daphnid population will consist almost entirely of females. Parthenogenesis is the predominant (i.e., default) mode of reproduction in these populations of daphnids. Environmental stimuli that indicate a change to adverse conditions signal the organisms to switch from parthenogenetic to sexual reproduction. Stimuli that have been reported to cause this switch include photoperiod, food quality or quantity, and crowding (Carvalho and Hughes, 1983; Kleiven *et al.*, 1992; Stross and Hill, 1965). The precise stimuli required to stimulate sexual reproduction may vary among clones of some species (Deng, 1996; Ferrari and Hebert, 1982; Larsson, 1991) and often, multiple stimuli are required for a species to initiate sexual reproduction (Kleiven *et al.*, 1992; Korpelainen, 1989). It is likely that different species and clones have evolved to respond to

specific stimuli or sets of stimuli that provide the greatest survival advantage for the specific region occupied by the organism.

Two major coordinated events occur during the sexual reproductive phase of daphnids: 1) the production of males and 2) the production of haploid eggs that must undergo fertilization for embryo development. Little is known of the neuro-endocrine factors that link the environmental stimuli to the ultimate physiological responses of male and haploid egg production. A neuropeptide, diapause hormone, has been shown to control embryonic diapause in some insects (Nijhout, 1994). Resting eggs of daphnids, the product of sexual reproduction, are in a state of diapause. Thus, a neuropeptide, similar to that identified in insects, may contribute to some aspects of the sexual reproductive cycle in daphnids. Adult diapause in insects is known to be directly affected by juvenile hormones. Application of juvenile hormones to diapausing females causes the return of reproductive activity in *Leptinotarsa decemlineata* (Schooneveld *et al.*, 1977) and *Pyrrhocoris apterus* (Hodkova, 1977).

Methyl farnesoate, the unepoxidated form of Juvenile Hormone III, functions as a gonadotropin in crustaceans and may be functionally homologous to juvenile hormone in insects (Homola and Chang, 1997; Laufer *et al.*, 1993). Based upon the demonstrated influence of methyl farnesoate on reproduction in decapod crustaceans, we hypothesized that this hormone may function in some aspect of reproduction in cladocerans and other lower crustaceans. The diminutive size of daphnids precludes administration of exogenous methyl farnesoate by injection. Thus, the role of terpenoid hormones in the sexual reproductive

cycling of daphnids was evaluated in the present study through aqueous exposure to the synthetic terpenoid hormone methoprene (11-methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid 1-methylethyl ester). Methoprene has adequate environmental stability for direct aqueous exposures, is readily accumulated by organisms, and elicits juvenile hormone activity in insects. For these reasons, methoprene is used commercially as an insecticide.

The specific hypothesis tested in this study was that terpenoid hormones regulate specific components of the cycling between asexual and sexual reproduction in daphnids and that these components could be discerned by the agonistic or antagonistic effects of the juvenile hormone analog methoprene.

METHODS AND MATERIALS

Daphnid (*Daphnia magna*) and algal (*Selenastrum capricornutum*) cultures were maintained as described previously (Baldwin and LeBlanc, 1994). The algae was used as a food source for daphnids during culturing and experimentation. Asexual reproduction was maintained by culturing daphnids at a density of 40 adult organisms in 1 L medium at a temperature and photoperiod of 20°C and 16 hr, respectively. Algae (1.4×10^8 cells) were provided to each 1 L culture twice daily and offspring were removed from the cultures at least three times weekly. Culture daphnids were nutritionally supplemented with a fish food homogenate, prepared as described previously (Baldwin and LeBlanc, 1994), and provided to the cultures at 4 mg (dry weight) twice daily.

Experiments were initially conducted to identify the environmental stimuli that triggered the switch from asexual to sexual reproduction in our clone of *D. magna*. The origin of this clone is not known. The culture was initiated from organisms derived from cultures maintained at the U.S. Environmental Protection Agency laboratory, Duluth, MN. The culture has been maintained in our laboratory for approximately 10 years. All experiments were initiated with neonatal daphnids (<24 hr old) and conducted at 20°C under a 16 hr photoperiod. In all experiments, culture media was changed every three days and offspring were removed daily. The effects of algal concentration (i.e. food deprivation) and crowding on reproductive strategy was assessed using the following experimental designs. The selection of food concentrations and daphnid densities used in these experiments were based upon preliminary experiments. *Uncrowded, high food concentration* (Treatment A): Daphnids were individually reared in 40 mL media and provided 3.5×10^6 cells algae twice

daily. *Uncrowded, low food concentration* (Treatment B): Daphnids were individually reared in 40 mL media and provided 1.0×10^6 cells algae twice daily. *Crowded, high food concentration* (Treatment C): Fifteen daphnids were reared in 200 mL media and provided 7.0×10^7 cells algae twice daily. *Crowded, low food concentration* (Treatment D): Fifteen daphnids were reared in 200 mL media and provided 2.1×10^6 cells algae twice daily. Sex of offspring was determined microscopically, by observations of the length of the first antennae, which in neonatal males are significantly longer than those of females (Olmstead and LeBlanc, 2000). Ehippia, highly pigmented protective structures that encase resting eggs (Hebert, 1978; Schultz, 1977), were counted and removed daily. Typically, two resting eggs are present within an ehippium and require fertilization for development. All experimental treatments were replicated 4 to 10 times. Experiments were conducted for 22 days.

Four nominal concentrations of methoprene (Chem Service, West Chester, PA, USA) were used in all treatment scenarios. These concentrations were known to be non-lethal based on previous work (Olmstead and LeBlanc, 2000) and are relevant to environmental concentrations. The use of methoprene at recommended application rates would be expected to result in environmental concentrations of ~ 30 nM (Ingersoll *et al.*, 1999). Methoprene concentrations in natural and experimentally enclosed surface waters following application at recommended rates have typically ranged from 3.0-30 nM (Knuth, 1989; Ross *et al.*, 1994). Methoprene was delivered in absolute ethanol. All test media had the same nominal ethanol concentration of 0.003 % v/v.

Data was analyzed using JMP software (SAS Institute, Cary, NC, USA). Bartlett's test for homogeneity of variance and Shapiro-Wilk W-test for normality was performed on the experimental data (Zar, 1996). Results involving several treatments were analyzed by ANOVA and Dunnett's t-test (Zar, 1996). In the cases where the data failed tests for homogeneity of variance and normality, a Kruskal-Wallis test was performed with the nonparametric multiple comparisons with control procedure of Dunn (Zar, 1996). Student's t-tests were used to compare the means of two treatments. Percentages were arcsin transformed before statistical analysis. Pearson's correlation coefficient (Zar, 1996) was calculated to evaluate the relationship between ehippia production and overall daphnid fecundity. Differences were deemed significant at an alpha level of 0.05.

RESULTS

Stimulation of the sexual reproductive phase by culture conditions

Initial experiments were performed to determine the ability of two culture conditions, crowding and food restriction, to stimulate entry of daphnids into a cycle of sexual reproduction. Food concentration had a direct impact on the number of parthenogenically produced offspring under either uncrowded or crowded conditions (Table 1, controls). Under uncrowded conditions, the percentage of male offspring produced was low with a high level of interindividual variability (Table 1, treatments A & B, controls). There was no significant difference in the percentage of male offspring produced and no discernible stimulation of ephippia production under uncrowded conditions provided food at either a high or low concentration.

Daphnids reared under high food and crowded conditions produced few males and no ephippia (Table 1, treatment C, control). However, the percentage of males produced increase 6-fold ($p < 0.001$) and ephippia were produced ($p < 0.001$) when daphnids cultured under crowded conditions were also provided food at a low concentration (Table 1, treatment D, control). These results demonstrated that our clone of daphnids will enter a sexual reproductive phase under conditions of crowding and food restriction.

Influence of methoprene upon entry into sexual reproduction

Experiments were next conducted to determine whether methoprene influenced entry into the sexual reproductive phase under any of the four combinations of crowding and food concentration. Methoprene had no significant effect on male or ephippia production under

crowding and food conditions that did not stimulate entry into the sexual reproductive phase (Table 1, treatments A, B, C). Methoprene did appear to stimulate male production in a concentration-dependent manner under uncrowded and low food conditions (Table 1, treatment B), though male production was statistically comparable to that of the control at all methoprene concentrations. Total parthenogenic reproduction was not significantly affected by methoprene for treatments B and C (Table 1). In treatment A, the 6.4 nM methoprene exposure group had significantly higher fecundity, while the 32 nM methoprene exposure group had significantly lower fecundity. Given these effects do not follow a typical concentration-response curve and are inconsistent with experiments performed previously (data not presented), we concluded that these results were artifacts and not biologically significant.

Methoprene did impact male and ephippia production under culture conditions that were permissive of sexual reproduction. Methoprene increased both the total number of parthenogenically produced offspring and the percentage of these offspring that were male in a concentration-dependent manner (Table 1, treatment D). Conversely, methoprene significantly reduced ephippia production in a concentration-dependent manner. Thus, under culture condition that are permissive of sexual reproduction, methoprene had multiple and apparently incongruous effects. Methoprene stimulated asexual reproduction as indicated by the concentration-dependent increase in the number of parthenogenetically-produced offspring. Simultaneously, methoprene stimulated one hallmark of sexual reproduction (male production) and suppressed another (ephippia production).

Influence of methoprene on the timing of events within the sexual reproductive phase

Sexual reproduction in daphnids is generally considered to proceed in a timed sequence of events beginning with the production of males followed by the production of resting eggs and ehippia (Hebert, 1978). Results from the present study demonstrated that methoprene had quantitatively different effects on male and ehippia production.

Experiments were next conducted to determine whether methoprene also influenced the sequence of male and ehippia production during the sexual reproductive phase.

Control daphnids produced the greatest percentage of males during the initial brood period (days 7-10) (Fig. 1). Average percentage male production among controls was less than 10% at all subsequent brood periods. Methoprene exposure (160 nM) significantly reduced the percentage of males produced during the initial brood period (Fig. 1) despite the increased production of offspring caused by this juvenoid (Fig. 2). Methoprene (32 or 160 nM) subsequently increased the percentage of male offspring produced at all brood periods. Thus, methoprene suppressed male production early in the sexual reproductive phase, when male production is normally high, and significantly increased the percentage of male offspring produced later in the sexual reproductive phase when male production is normally low.

Methoprene suppressed ehippia production as observed in earlier experiments but had no discernible effect on the temporal production of ehippia (Fig. 3). Considering the inverse relationship between methoprene's stimulation of the number of parthenogenetically produced neonates (Fig. 2) and the number of resting egg-containing ehippia (Fig. 3), we

hypothesized that methoprene suppressed the production of broods of haploid eggs and, as a result, the daphnids produce more broods of diploid parthenogenetic offspring. A significant inverse relationship did exist between the total number of offspring produced by daphnids exposed to the various methoprene concentrations and the number of ephippia produced by these organisms (Fig. 4). These observations suggest that methoprene reduced the number of broods of “sexual” resting eggs which were supplanted by broods of asexually-produced offspring.

DISCUSSION

The hypothesis tested in this study was that terpenoid hormones regulate specific components of the cycling between asexual and sexual reproduction in daphnids and that these components could be discerned by the agonistic or antagonistic effects of the juvenile hormone analog methoprene. Results clearly demonstrate that methoprene can indeed modulate specific components of the sexual reproductive phase of daphnids. The low concentrations at which methoprene elicited these effects support the hypothesis that this juvenoid was acting at specific receptors to endogenous juvenoids.

Methoprene was not able to stimulate entry into the sexual reproductive phase of the daphnids. Such a property of methoprene would have been evident by a consistent increase in male and ephippia production with increasing methoprene exposure concentration under the various experimental designs (Table 1). Rather, entry into the sexual reproductive phase was stimulated by the combination of crowding and food restriction. One could argue that food restriction is the single determinant of entry into the sexual reproductive phase and that crowding simply exacerbated food restriction. However, fecundity of daphnids reared under uncrowded or crowded conditions with food restriction produced comparable numbers of offspring. This indicates that individual daphnids in both treatment groups experienced similar nutritional limitation. Yet, only daphnids reared under the crowded conditions produced significant numbers of males and ephippia. Thus, the combination of food restriction and crowding conditions, but not methoprene, stimulated entry of our clone of daphnids into the sexual reproductive phase. We postulate that these environmental stimuli initiate a neuro-endocrine cascade, that does not involve juvenoids, which initiates entry into

the sexual reproductive phase. A neuropeptide such as the diapause hormone identified in some insects (Nijhout, 1994) may be primarily responsible for entry of daphnids into the sexual reproductive phase.

Upon entry into the sexual reproductive phase, daphnids produce males (Hebert, 1978) (Fig. 1). Additional males are subsequently produced along with broods of parthenogenetically-produced (diploid) females and haploid eggs. Resting eggs generally require fertilization and undergo embryonal diapause prior to hatching (Alekseev and Starobogotov, 1996). The production of males and resting eggs is time coordinated (Pennak, 1953) and the appropriate sequence of these events presumably maximizes the production of fertile resting eggs that perpetuate the population through adverse environmental conditions. Results from the present study suggest that juvenoids are involved in regulating the sequence of events during the sexual reproductive phase and that the juvenoid analog methoprene can interfere with the temporal regulation of these events. Methoprene a) reduced male production during the early stage of the sexual reproductive phase when male production is normally highest, b) stimulated male production during latter stages in the cycle, when male production is normally low, and c) stimulated the production of parthenogenetic broods of daphnids at the expense of resting egg production. These effects were elicited at low nominal methoprene exposure concentrations suggesting that these effects were the consequence of methoprene's interactions with high affinity receptors rather than some overt toxicological response.

Previous studies have demonstrated that exposure to some chemicals can influence the number of males produced by daphnids once entered into the sexual reproductive phase. Atrazine and 4-nonylphenol have been shown to stimulate male production (Baer and Owens, 1999; Dodson *et al.*, 1999a). Dieldrin exposure reduced the number of males produced by daphnids (Dodson *et al.*, 1999b). The mechanism by which these chemicals influence male production is not known. Results from the present study suggest that these chemicals may be interfering with normal activity of endogenous juvenoid hormones.

Results of this study, in combination with previous studies of diapause in arthropods, allows for the proposal of a testable model for the endocrine regulation of sexual reproduction in daphnids. We propose that environmental stimuli (food deprivation and crowding in the case of our clone of *D. magna*) stimulates a neuro-endocrine response (i.e., secretion of the neuropeptide diapause hormone (Nijhout, 1994)). The neuropeptide triggers entry into the sexual phase of the reproductive cycle and alters the responsiveness of the organisms to terpenoid hormone (i.e., methyl farnesoate). Once entered into the sexual phase, terpenoid hormone functions, in combination with other regulators to coordinate the production of males and resting eggs in some reciprocal fashion.

An example scheme for the action of juvenoid in combination with two other regulators (designated X and Y) in the coordinated regulated of male and resting egg production is depicted in Table 2. According to this scheme, low levels of endogenous juvenoid (i.e., methyl farnesoate) are permissive for the production of either parthenogenetically-produced males, parthenogenetically-produced females, or resting eggs.

Combinations of factors X and Y dictate whether the daphnid brood will consist of males, females, or resting eggs. High juvenoid levels allow only for parthenogenetic reproduction with the production of female or male broods dictated by factor Y. According to this paradigm, methoprene would influence juvenile hormone-regulated processes by acting as an agonist when endogenous juvenoid levels are low. Thus, methoprene could either inhibit (as observed during the initial brood period) or stimulate (as observed during subsequent brood periods) male production. Methoprene could also favor the production of parthenogenetic broods at the expense of resting eggs (as depicted in Fig. 4). This paradigm serves to exemplify how methoprene could uncouple male and resting eggs production. Further studies are required to definitively identify the mechanism by which this uncoupling occurs.

Alterations in the sequence of male and resting eggs production by methoprene raises some relevant questions regarding the potential population-level effects of this pesticide. For example, if methoprene exposure delays male production, what consequence would this have on the fertilization of resting eggs that may be necessary for the population to survive winter or other environmental adversity? Methoprene exposure would be predicted to increase the relative abundance of males later in the reproductive cycle. Would methoprene compromise the optimum ratio of males and sexually receptive females required for adequate production of diapause eggs? Questions such as these raise the possibility that insect growth regulating pesticides may elicit effects on some non target populations (i.e., Cladocera) in a manner and at exposure levels not anticipated by standard toxicity evaluations using only parthenogenetically-reproducing organisms.

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Table 1. Ability of combinations of food restriction, crowding, and methoprene exposure to stimulate sexual reproduction in daphnids (*Daphnia magna*). Entry into the sexual reproductive phase was discerned by the production of males and resting egg-containing ephippia. Experiments were conducted for 22 days. Food concentrations and crowding conditions are described in the Materials and Methods section. Treatments A: uncrowded, high food concentration; B: uncrowded, low food concentration; C: crowded, high food concentration; D: crowded, low food concentration. All data are presented as a mean plus or minus the standard deviation.

<i>Treatment</i>	<i>Methoprene</i> (nM)	<i>Offspring/</i> female	<i>Male (%)</i>	<i>Ephippia/</i> female	<i>n</i>
A	Control	61 ± 5	7 ± 16	0	10
	1.3	64 ± 6	2 ± 8	0	10
	6.4	73 ± 6*	6 ± 11	0	7
	32	53 ± 5*	12 ± 19	0	6
	160	60 ± 6	7 ± 12	0	7
B	Control	13 ± 2	16 ± 16	0	10
	1.3	11 ± 2	3 ± 9	0	7
	6.4	12 ± 3	17 ± 16	0	9
	32	12 ± 4	28 ± 24	0.1 ± 0.3	9
	160	14 ± 2	31 ± 32	0	8

Table 1 (continued)

C	Control	83 ± 6	2 ± 1	0	4
	1.3	87 ± 8	3 ± 2	0	4
	6.4	86 ± 4	3 ± 2	0	4
	32	85 ± 4	0 ± 1	0	4
	160	84 ± 6	1 ± 1	0	4
D	Control	7 ± 1	12.1 ± 4.2**	1.25 ± 0.23**	8
	1.3	8 ± 1	12.1 ± 5.4	1.33 ± 0.20	9
	6.4	8 ± 1	18.8 ± 7.5	0.81 ± 0.30*	9
	32	13 ± 1*	21.6 ± 6.4*	0.38 ± 0.17*	9
	160	13 ± 1*	24.5 ± 8.4*	0.37 ± 0.21*	9

*Significantly different from the respective control at $P \leq 0.05$.

**Significantly different from Treatment C control at $P \leq 0.001$.

Table 2. Paradigm for the uncoupling of male and resting egg production by methoprene. “Juvenoid”, “X”, and “Y” represent three postulated endogenous regulators involved in the coordination of sexual reproduction. Plus and minus symbols represent the presence of the factors at either high or low levels.

Juvenoid	X	Y	Production	Methoprene	Response
-	-	+	parthenogenesis: male	agonist	decreased male broods, increased female broods
-	+	+	resting eggs, female	agonist	increased parthenogenesis: females, decreased resting eggs
-	+	-	parthenogenesis: females	agonist	decreased female broods, increased male broods
+	-	+	parthenogenesis: females	no effect	none
+	+	+	parthenogenesis: females	no effect	none
+	+	-	parthenogenesis: males	no effect	none

FIGURE LEGENDS

Figure 1. Percentage of male offspring produced by daphnids exposed to concentrations of methoprene under conditions of crowding and food restriction. Data are presented as the mean \pm standard deviation (n=9) number of offspring produced during five time intervals. Treatments are: control (□), 1.3 nM methoprene (■), 6.4 nM methoprene (▨), 32 nM methoprene (▩), 160 nM methoprene (▧). An asterisk indicates a significant ($P \leq 0.05$) difference from the control.

Figure 2. Number of offspring parthenogenetically-produced by daphnids exposed to concentrations of methoprene under conditions of crowding and food restriction. Data are presented as the mean \pm standard deviation (n=9) number of offspring produced during five time intervals. Treatments are: control (□), 1.3 nM methoprene (■), 6.4 nM methoprene (▨), 32 nM methoprene (▩), 160 nM methoprene (▧). An asterisk indicates a significant ($P \leq 0.05$) difference from the control.

Figure 3. Ehippia production by daphnids exposed to concentrations of methoprene under conditions of crowding and food restriction. Data are presented as the mean \pm standard deviation (n=9) number of offspring produced during five time intervals. Treatments are: control (□), 1.3 nM methoprene (■), 6.4 nM methoprene (▨), 32 nM methoprene (▩), 160 nM methoprene (▧). An asterisk indicates a significant ($P \leq 0.05$) difference from the control.

Figure 4. Relationship between ephippia and offspring production by daphnids exposed to concentrations of methoprene under conditions of crowding and food restriction. Each data point represents the performance of daphnids (n=9) in one replicate vessel. Treatments are: control (▲), 1.3 nM methoprene (◆), 6.4 nM methoprene (+), 32 nM methoprene (×), 160 nM methoprene (●). Pearson's correlation coefficient was -0.89 ($P < 0.001$).

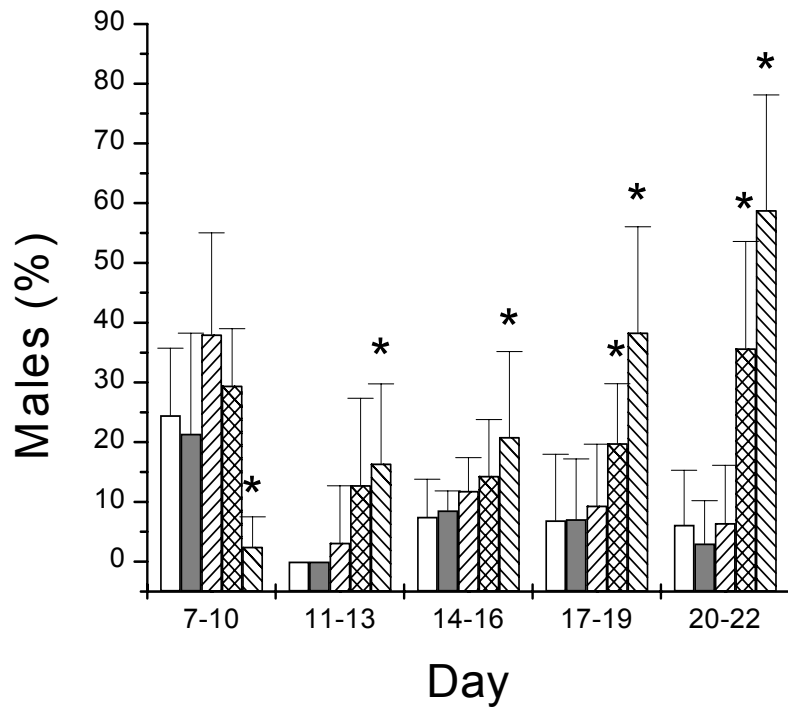


Figure 1

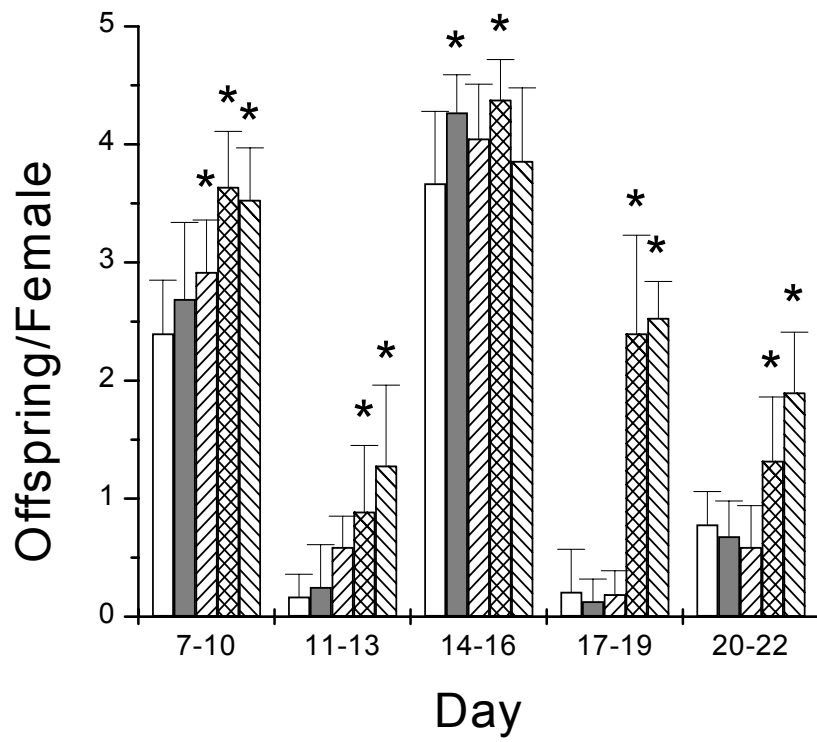


Figure 2

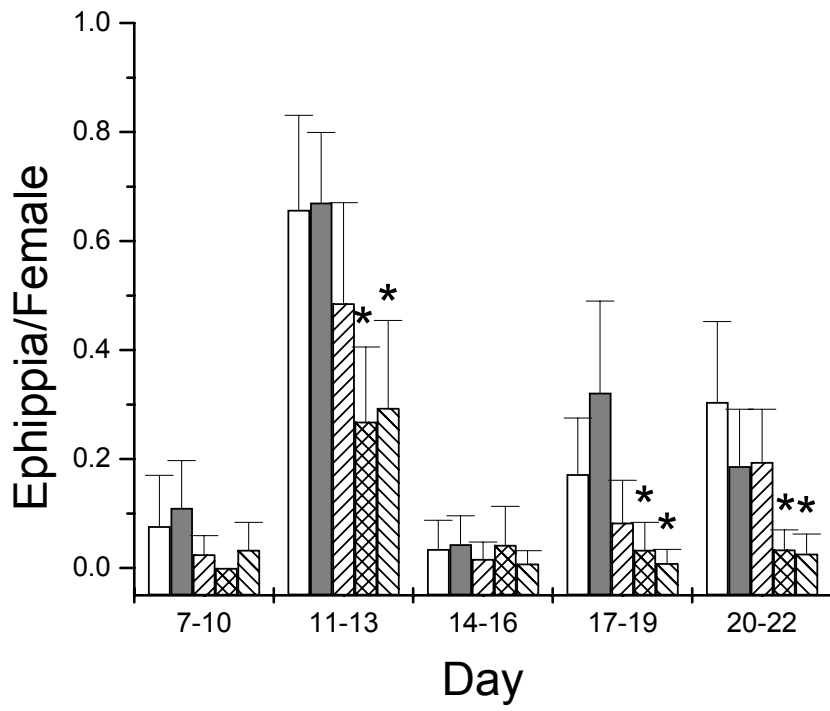


Figure 3

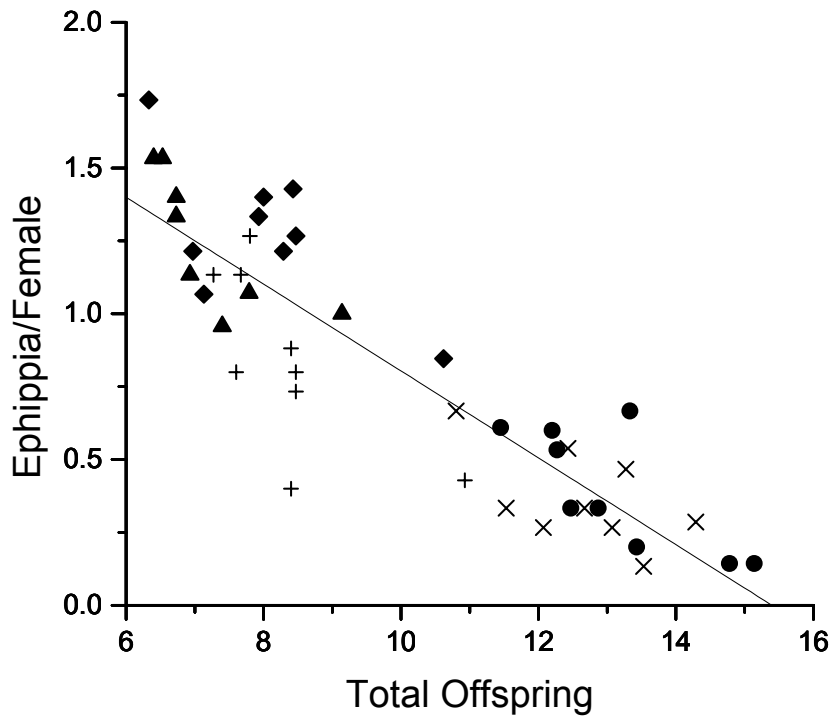


Figure 4

**The Juvenoid Hormone Methyl Farnesoate is a Sex
Determinant in the Crustacean *Daphnia magna***

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pp. 736-739) and is in the format required of that journal.

ABSTRACT

Daphnids (*Daphnia magna*) utilize cyclic parthenogenesis as a reproductive strategy. During periods of abundant resources, these organisms reproduce asexually. In response to environmental cues that signal the onset of environmental adversity, daphnids produce males and reproduce sexually. The environmental cues that stimulate the sexual reproductive phase are well known; however, the endocrine signals that transduce these environmental cues remain unknown. The present study was undertaken to test the hypothesis that the crustacean juvenoid hormone, methyl farnesoate, is a male sex determinant in this species. Continuous exposure to aqueous concentrations of methyl farnesoate greater than approximately 30 nM stimulated a concentration-dependent production of male-containing broods of organisms. Short-term exposures to methyl farnesoate during periods of egg and embryo maturation revealed that male sex determination occurred during a specific 12-hour period of ovarian egg development. Exposure of eggs to 400 nM methyl farnesoate during this sensitive developmental period resulted in the production of all-male broods of offspring, while exposure to concentrations as low as 52 nM produced mixed broods of males and females. This active concentration range of methyl farnesoate is consistent with levels measured in the hemolymph of some decapod crustaceans. These results demonstrate that methyl farnesoate is capable of programming daphnid embryos to develop into males and is likely the endocrine factor responsible for initiating the sexual reproductive phase in these organisms.

INTRODUCTION

Water fleas (Cladocera, Crustacea) utilize dual reproductive strategies during their life history. During periods of abundant resources, water fleas reproduce by parthenogenesis with females producing broods of genetically identical diploid off-spring (Hebert, '78; Lynch and Gabriel, '83). These offspring are typically all females and, assuming maintenance of ample resources, will develop into parthenogenically-reproducing females that continue to produce all-female broods. Under this reproductive scenario, the water flea population can rapidly expand and fully exploit a favorable environment. Under conditions of diminishing resources, adult females will partheno-genically produce broods of male offspring (Hebert, '78). These males mature and mate with receptive females that have produced a limited number of haploid eggs. These fertilized eggs are encased in a protective ephippium and can exist in a diapause state until favorable conditions return (Hebert, '78; Grebelnyi, '96).

Multiple environmental signals have been shown to stimulate the entry of water fleas into the sexual reproductive phase beginning with the production of males. These environmental stimuli include reduced photoperiod, reduced food quantity and quality, and crowding (Stross and Hill, '65; Carvalho and Hughes, '83; Kleiven et al., '92). Different species and different clones within a species have been shown to respond to different environmental stimuli (Ferrari and Hebert, '82; Larsson, '91; Deng, '96). We have shown that the clone of daphnids (*Daphnia magna*) used in our laboratory produces males in response to a combination of crowding and reduced food quantity (Olmstead and LeBlanc, 2001b). Endocrine factors responsible for transducing environmental cues for the initiation of the sexual reproductive phase are not known.

Methyl farnesoate (MF), the unepoxidated form of juvenile hormone III of insects, is produced by the mandibular organ in crustaceans and appears to regulate many aspects of reproduction including the stimulation of male morphogenesis. For example, MF levels in the hemolymph of the spider crab (*Libinia emarginata*) are positively correlated to claw size of males (Sagi et al., '93), a male secondary sex characteristic. MF stimulates testicular growth in the freshwater crab (*Oziotel-phusa senex senex*) (Kalavathy et al., '99). Taken together, these observations indicate that MF functions to stimulate male morphogenesis and raises the question as to whether MF may also function as the endocrine signal that is responsible for male sex determination in cyclic parthenogens such as daphnids. In the present study, we have demonstrated that MF indeed causes adult parthenogenically-reproducing daphnids to switch from the production of female to the production of male offspring.

MATERIALS AND METHODS

The ability of MF to modulate the sex of daphnid offspring was first evaluated by chronically exposing daphnids to 90 different concentrations of MF (73% all-trans, 27% cis, trans) ranging from 0.0065 nM to 800 nM for 21 days using methods as described previously (Olmstead and LeBlanc, 2001a). These daphnids were < 24 hours old at the initiation of the exposures and each organism had parthenogenically produced 3–5 broods of offspring by the end of the experiment. Experimental conditions were selected to promote the asexual production of female offspring. Sex of neonates was determined by microscopic examination of the primary antennae (Olmstead and LeBlanc, 2000). Solutions of MF were initially prepared in absolute ethanol and the final concentration of ethanol in all test solutions, including controls, was 0.003%. Ethanol, at this concentration, had no effect on male production.

Next, experiments were conducted to determine the precise period during egg/embryo development during which MF dictated sex of the offspring. The development of a brood of eggs/embryos in *D. magna* occurs over two molt cycles (approximately six days). During the first intermolt period, egg maturation occurs in the ovaries. Following molting, a brood of eggs is transferred from the ovary to the brood chamber where embryo development occurs. Upon completion of embryo development, fully developed offspring are released from the mother just prior to the next molt.

Eighty adult female daphnids (ten days old) were individually and randomly assigned to beakers containing culture media (Olmstead and LeBlanc, 2000). Beakers were divided

into groups of ten, and daphnids in each group would subsequently be exposed to MF during different 24-hour periods. Beakers were examined every 12 hours for the presence of a molted exoskeleton. The time at which the exoskeleton was observed was designated as the beginning of the intermolt period (0 hour) during which ovarian egg development occurred. Each daphnid was then exposed to 200 nM MF at its designated exposure period. The first treatment group was exposed to MF from 0 to 24 hours, the next from 12 to 36 hours, etc.(see Fig. 1B). Each exposure interval overlapped by 12 hours with the adjacent exposure periods. Finally, eggs were exposed to concentrations of MF only during the most sensitive 24 hour period of ovarian egg development, as determined from the previous experiment (48–72 hrs), and sex outcome of the broods was determined.

RESULTS AND DISCUSSION

Continuous exposure of daphnids to concentrations of MF over a 21-day period provided clear evidence that MF stimulated the production of male progeny in this species (Fig. 1A). Only female offspring were produced among daphnids exposed to concentrations of MF < 30 nM. Daphnids progressively produced greater numbers of male-containing broods with increasing MF concentrations > 30 nM.

Next, experiments were performed to identify the period during egg/embryo development during which daphnids are susceptible to sex determination by MF. Male sex of offspring was determined by MF during the egg maturation period and not during embryo development, as none of the embryos exposed to MF while in the brood chamber of the maternal organisms developed into males (data not shown). Only offspring exposed to MF during ovarian egg maturation were male. Furthermore, the greatest percentage of male-containing broods (490%) occurred among eggs exposed to MF during the 60–72 hour period of ovarian development (Fig. 1B).

Exposure of daphnids to concentrations of MF only during the 24-hour period of egg maturation that included the period of sensitivity to the hormone produced a concentration-dependent incidence of male-containing broods that was consistent with the previous chronic exposure (Fig. 1C versus 1A). This active concentration range (31–400 nM) is remarkably similar to levels measured in the hemolymph of three species of crabs (40–240 nM) (Borst et al., '87). Some mixed male/female broods were noted at the lower end of the concentration-response curve (52–144 nMMF), while all males were produced at the highest MF treatment

level (400 nM). This level of potency of MF, coupled with the observation that MF is present in branchiopods (Laufer and Biggers, 2001), strongly suggests that MF is an active hormone in daphnids.

These results demonstrate that, in addition to stimulating the development of secondary sex characteristics in crustaceans (i.e., Kalavathy et al., '99), MF can actually program parthenogenic eggs of *D. magna* to develop into males. Thus, MF is a likely endocrine signaling factor that is responsible for initiating a sexual reproductive phase in response to environmental signals in this species. We previously had shown that exposure of daphnids to the juvenile hormone analog methoprene alone did not stimulate male production. However, this MF-like compound did stimulate male production in daphnids that had been primed for a sexual reproductive cycle through the provision of environmental stimuli (Olmstead and LeBlanc, 2001b). Thus, methoprene may function as a weak MF agonist that requires ancillary support to elicit a full MF-like response. These observations raise the possibility that other juvenile hormone analogs that are commercially used as insecticides may actually alter sex ratios of daphnids and other cyclic parthenogenic arthropods at environmentally relevant concentrations.

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FIGURE LEGEND

Fig. 1. Stimulation of male production among daphnids (*Daphnia magna*) by methyl farnesoate (MF). A. Concentration-response relationship during continuous treatment with MF. Daphnids were individually exposed to 90 concentrations of MF through their entire life cycle. Each data point represents the percentage of broods containing males released by one daphnid. Data were transformed to best define the concentration-response curve using the moving average method (Microcal Origin, Northampton, MA). B. Identification of the period of susceptibility to the sex-determining effects of MF. Female daphnids (ten per exposure period) were exposed to 200 nM MF during the indicated periods of egg maturation, and sex of the individuals in the resulting broods of offspring was determined. Each bar represents the percentage of male-containing broods from ten daphnids. Controls represent unexposed daphnids. C. Concentration-response relationship from treatment with MF only during the sensitive period of egg maturation (48–72 hr after initiation of the egg maturation intermolt period). Experiments were performed as described under ‘B’ except that MF treatment level was the variable and treatment period was held constant.

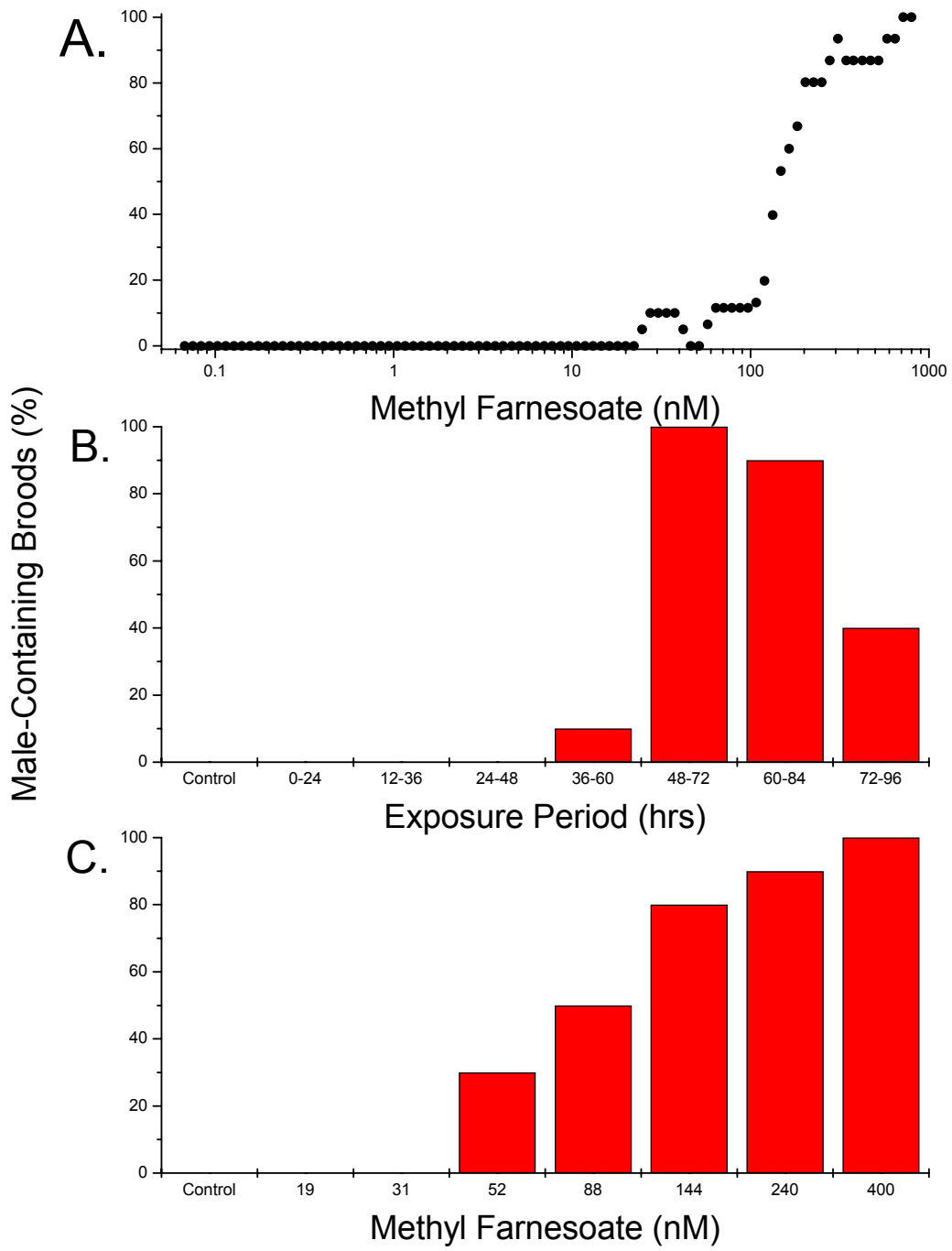


Figure 1

**Insecticidal Juvenile Hormone Analogs Stimulate the
Production of Male Offspring in the Crustacean,
*Daphnia magna***

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ABSTRACT

Juvenile hormone analogs (JHAs) represent a class of insecticides that were specifically designed to disrupt endocrine-regulated processes relatively unique to insects. Recently, we demonstrated that the crustacean juvenoid hormone, methyl farnesoate, programs oocytes of the crustacean *Daphnia magna* to develop into males. We hypothesized that insecticidal JHAs might mimic the action of methyl farnesoate resulting in altered sex ratios of offspring. Daphnids were chronically exposed (3 weeks) to sublethal concentrations of methyl farnesoate, the JHA pyriproxyfen, and several chemicals that are known to elicit stress through various non-juvenoid mechanisms in order to discern whether excess male offspring production is a generic response to stress or a specific response to juvenoid hormones. Only methyl farnesoate and pyriproxyfen increased the percentage of males produced by exposed maternal organisms. As previously reported with methyl farnesoate, acute exposure (24 hours) to either pyriproxyfen or the JHA methoprene caused oocytes maturing in the ovary to develop into males. Experiments were performed to determine whether combined effects of a JHA and methyl farnesoate better conformed to a model of concentration addition (indicative of same mechanism of action) or independent joint action (indicative of different mechanisms of action). Combined effects conformed better to the concentration addition model though some synergy, of unknown etiology, was evident between the insecticides and the hormone. These experiments demonstrate that insecticidal JHAs mimic the action of the crustacean juvenoid hormone methyl farnesoate resulting in the inappropriate production of male offspring. The occurrence of such an effect in the environment could have dire consequences on susceptible crustacean populations.

INTRODUCTION

Environmental sex determination occurs in a species when the sex of offspring is determined by prevailing environmental conditions (Korpelainen 1990). Species exhibiting this form of sex determination can be found among rotifers, nematodes, polychaetes, crustaceans, insects, fish, and reptiles (Korpelainen 1990). In all cases of environmental sex determination, animals interpret cues from the environment that indicate whether male or female progeny would have greater reproductive fitness and increase population sustainability. Hormonal or metabolic pathways are altered in receptive individuals in response to the environmental stimuli that lead to the production of offspring of the desired sex. Human activity, including the introduction of xenobiotics into the environment can disrupt this process. For example, exposure of turtle eggs to some polychlorinated biphenyls can skew sex ratios of offspring in favor of females (Bergeron, et al. 1994).

The crustacean, *Daphnia magna* exhibits environmental sex determination. Broods of female offspring are produced under favorable environmental conditions and the daphnid population expands through asexual (parthenogenic) reproduction. When environmental cues, such as shortening of the daylight period and decreased food occur, these populations begin to produce males and undergo a cycle of sexual reproduction (Hebert 1978). Sexual reproduction results in the generation of dormant resting eggs encased in protective ephippia. These resting eggs will resume development and hatch when environmental conditions improve, allowing a population to survive in a habitat that periodically becomes inhospitable to the adult (Hebert 1978, Wolf and Carvalho 1989).

While the environmental stimuli that induce the production of male progeny in daphnid populations have been well studied, the endocrinology of this event has not been fully characterized. We have recently reported that exposure of daphnid oocytes to the crustacean hormone, methyl farnesoate, during late ovarian development causes the oocytes to develop into males whereas only females are produced in unexposed animals (Olmstead 2002). Thus, methyl farnesoate is a likely endocrine factor that transduces the environmental cues (changes in photoperiod, reduced food, etc.) to the physiological response (production of male offspring). Methyl farnesoate is a terpenoid hormone synthesized by the mandibular organ in decapod crustaceans (Ding 1991, Borst 1994) and is involved in various aspects of crustacean reproduction and juvenile development (Homola 1997, Laufer 1993, Laufer 2001). We propose that methyl farnesoate stimulates male progeny production by activating as an ultraspiracle-like receptor in daphnids. Ultraspiracle is an invertebrate retinoid X receptor ortholog found in insects and crustaceans (Oro 1990, Chung, 1998). Methyl farnesoate is structurally very similar to the insect terpenoid Juvenile Hormone III (Figure1).

In a previous study, we reported that chronic exposure of daphnids to the insecticidal juvenile hormone analog (JHA) methoprene shifted sex ratios of offspring towards males when compared to controls (Olmstead and LeBlanc 2001). We hypothesized that methoprene elicits this effect on daphnid sex determination by acting as a methyl farnesoate agonist. We tested this hypothesis in the present study by exposing daphnids to a variety of chemicals to determine if male induction was unique to JHAs. We also evaluated the ability of the JHAs methoprene and pyriproxyfen to program sex in oocytes during late ovarian maturation as was observed with methyl farnesoate (Olmstead and LeBlanc 2002). Finally, binary

combinations of the JHAs and methyl farnesoate were evaluated for concentration additivity as an indicator of shared mode-of-action. This model is similar to the toxic equivalency approach used to assess the combined toxicity of chemical mixtures (i.e. dioxins) that elicit effects through a common mechanism (ref). However, if the JHAs and methyl farnesoate alter sex ratios through different mechanisms, then their combined effects would conform with a model for independent joint action (Bliss 1939). This model assumes that the two chemicals elicit a common effect (altered sex ratios) by acting at different sites along the signaling pathway leading to sex determination.

METHODS AND MATERIALS

Daphnid culture

Daphnids (*Daphnia magna*) were cultured in incubators at a density of 40 adults in 1 L medium at a temperature and photoperiod of 20⁰C and 16 hr light. Algae (*Selenastrum capricornutum*), cultured in Bold's Basal medium, was used as a food source for daphnids during culturing and experimentation. Algae (1.4×10^8 cells) were provided to each 1-L culture twice daily and offspring were removed from the cultures at least three times weekly. Cultures were nutritionally supplemented with a fish food homogenate, prepared as described previously (Baldwin and LeBlanc 1994) and provided to the cultures at 4 mg (dry weight) twice daily. Cultured daphnids reproduce asexually under these conditions with virtually all progeny (>95%) being female.

Male progeny production during chronic exposure

We had previously determined that the strain of daphnids used in our laboratory enters a sexual reproductive phase in response to high population density and reduced food availability (Olmstead and LeBlanc 2001). We also demonstrated that culturing of daphnids under environmental conditions that permitted a basal level of male progeny production allowed for increased male production upon exposure to the juvenile hormone analog, methoprene (Olmstead and LeBlanc 2001). Therefore, daphnids were exposed to various chemicals under conditions of high population density (15 daphnids in 200 mL of media) and low food level (2.1×10^6 cells provided twice daily) and the stimulation of male progeny production was evaluated.

Experiments were initiated with neonatal daphnids (<24 hours old) and proceeded through approximately four brood cycles (21 days). Four treatment levels were evaluated for every chemical and each treatment was replicated nine times. Test solutions were maintained at 20⁰C under a 16 hr light photoperiod. Solutions were changed and offspring removed every three days. Sex of individual offspring was determined microscopically (10X magnification) with males being discerned from females by the longer primary antennae (Olmstead and LeBlanc 2000).

Several chemicals were evaluated for their ability to stimulate male progeny production. Exposure concentrations for each chemical were within the range of concentrations that impacted parthenogenic reproduction in standard life cycle tests. The pesticidal juvenile hormone mimic pyriproxyfen (Chem Service, West Chester, PA, USA) was evaluated to further test our hypothesis that this class of compounds specifically stimulates male progeny production through its action as a methyl farnesoate agonist. Methyl farnesoate (synthesized by Dr. M. Feldlaufer, USDA, Beltsville, Maryland and provided by Professor Huw H. Rees and Dr. Geoff Wainwright, University of Liverpool, Liverpool, UK) was used as the positive control (Olmstead 2002). The herbicide atrazine (Chem Service) was evaluated because this chemical was reported previously to stimulate male progeny production (Dodson, et al. 1999a). Fenarimol (Chem Service) was selected because this fungicide functions as an antiecdysteroid (Mu 2002) and ecdysteroids have been implicated in male progeny production (Peterson 2001). Pentachlorophenol (Chem Service), a polar narcotic and an uncoupler of oxidative phosphorylation (Schuurmann 1997), was used to determine whether male progeny production occurs in response to general metabolic

stress. Ethanol (Aaper, Shelbyville, KY, USA) was assessed because this alcohol was used as a carrier solvent for the other chemicals and its potential effect on male production required evaluation. All chemicals (except ethanol) were dissolved in ethanol as a carrier solvent. The concentration of carrier present in any given test solution never exceeded 0.005 % v/v. Control solutions contained the same concentration of ethanol as was present in the respective chemical treatments. Significant differences ($p \leq 0.05$) among treatments were evaluated using ANOVA and Dunnett's t test (JMP software, SAS Institute, Cary, NC, USA).

Exposure to JHAs during oocyte development

Methyl farnesoate was previously shown to program maturing oocytes in the ovary to develop into males (Olmstead 2002). Following ovarian maturation, the oocytes are transferred to the brood chamber of the maternal organism where the embryos develop. Free-swimming neonates are released from the brood chamber upon completion of embryo development. The transfer of oocytes from the ovaries to the brood chamber coincides with the molting of the maternal organism's exoskeleton and release of neonates from the brood chamber coincides with the next molt. Maternal daphnids were exposed to concentrations of pyriproxyfen, methoprene, or methyl farnesoate during oocyte maturation and sex of the resulting progeny exposed in the ovary was determined. Should pyriproxyfen and methoprene program sex of daphnids via the same mechanism as methyl farnesoate, then sex determination should occur during the same window of susceptibility.

Adult female daphnids, carrying embryos in their brood chambers, were selected from the cultures and placed individually in 50 mL beakers containing 40 mL of media. Beakers were examined every 12 hours for the presence of a molted exoskeleton. Forty-eight hours after detecting a molted exoskeleton, the daphnid was transferred to test media containing the appropriate concentration of the test chemical. The daphnid was maintained in this solution for 24 hours, which encompassed the sex-determining period of ovarian oocyte maturation. Daphnids then were transferred to juvenoid-free medium and maintained until the brood of offspring exposed to the juvenoid in the ovary was released. Food (*S. capricornutum*; 7×10^6 and fish food homogenate, 0.2 mg dry weight) was provided to each beaker twice daily. Daphnids typically produce only female offspring under these culture conditions. Sex of individual offspring was determined as described above. Results from these experiments were fitted to concentration-response curves with Origin software (MicroCal Software Inc., Northampton, MA, USA) using the following concentration-response equation:

$$R = \frac{100}{1 + 10^{(\log(EC_{50}) - \log(C_x))p}} \quad \text{Eq. 1}$$

The concentration of a given chemical x is denoted by C_x , p is the power or slope of the curve, and the response caused by exposure to that chemical is R, namely the percentage of males per brood. EC_{50} values for each chemical were determined from these fitted equations.

Binary combinations

A zero interaction concentration response surface for the concentration additive model was generated using the fitted concentration response curves generated for the

individual chemicals. This surface is the theoretical concentration-response surface for mixtures of two chemicals if they combine in a simple concentration-additive fashion (Gessner 1995, Poch 1993). The equation used to generate this curve was derived from Eq. 1 by adjusting the second chemical's concentration (C_y) with a relative potency factor ($EC50_x/EC50_y$) that expresses it in equivalencies of the first chemical (C_x):

$$R_c = \frac{100}{1 + 10^{(\log EC50_x - \log [C_x + C_y * EC50_x / EC50_y])p}} \quad \text{Eq. 2}$$

where R_c is the combined response of chemicals x and y; and C_x and C_y are the concentrations of chemicals x and y. The power of this curve, p' , is the average of the slopes from the individual concentration-response curves of the two chemicals. The independent joint action model (Bliss 1939) was generated with the following equation which is derived from probability theory:

$$R_c = R_x + R_y - R_x R_y \quad \text{Eq. 3}$$

where R_x and R_y are the responses for the individual chemicals x and y, respectively.

Various combinations of pyriproxyfen and methyl farnesoate or methoprene and methyl farnesoate were then experimentally evaluated for the stimulation of male progeny production using the same methods as used with the individual chemicals and described in the previous section. Model predictions of male offspring production were then generated for each chemical combination using the concentration additivity model and the independent joint action model. Model predictions were compared to actual results by calculation of coefficients of determination (r^2) (Zar 1996). The model producing the highest coefficient of determination best represented the experimental results.

RESULTS

Increased male progeny production from chemical exposure

Six chemicals were evaluated for their ability to stimulate male progeny production among daphnids. Only the juvenoid hormone methyl farnesoate and the JHA pyriproxyfen altered sex ratios of offspring in favor of males (Fig. 2). Under these exposure conditions, pyriproxyfen was two to three orders of magnitude more potent at stimulating male progeny production than was methyl farnesoate. Altered sex ratios were not due to differential embryo mortality since the total number of offspring produced among daphnids exposed to either methyl farnesoate or pyriproxyfen was not significantly different from the controls (data not shown). These results demonstrate that increased male production is not a generalized response of daphnids to chemical stress.

Male-sex determination during oocyte exposure

Experiments were performed to determine whether, like methyl farnesoate, JHAs determined the sex of daphnids during ovarian oocyte maturation. Maternal daphnids were exposed to the juvenoids under conditions that promoted the production of only female offspring. The sex of offspring that were present, as oocytes, in the ovaries of the maternal daphnids during juvenoid exposure were determined. Exposure of oocytes to methyl farnesoate during ovarian development programmed the oocytes to develop into male offspring in a concentration dependent manner (Fig. 3A) with an EC_{50} of 87 nM. Pyriproxyfen stimulated male progeny production among oocytes during ovarian development (Fig. 3B) with an EC_{50} of 0.31 nM. Methoprene also stimulated oocytes to develop into males, but only at much higher exposure concentrations (EC_{50} of 1140 nM).

The EC₅₀ value for male production by methoprene was approximately the concentration that is lethal to 50% of exposed neonatal daphnids (LC₅₀ = 1160 nM). Like methyl farnesoate, the JHAs program sex in oocytes during ovarian development.

Binary combinations

Results from the concentration-response analysis with the individual chemicals (Fig. 3) were used to model the zero-interaction concentration-response surfaces for binary mixtures of pyriproxyfen-methyl farnesoate and methoprene-methyl farnesoate. These models are presented as contour plots (Fig. 4) to illustrate differences between the concentration addition and independent joint action model. The greatest difference between model predictions was in the shape of the contour lines across the surface. Contour lines were straight along the entire response surface when using the concentration addition model (Figs. 4A, 5A) and in the independent joint action model contour lines were concave (Figs. 4B, 5B). The concave character of the contour plot generated with the independent joint action model indicates combined effects predicted with this model are less than those predicted by simple concentration additivity.

Similar contrasts between the shape of the response surface were evident with the methoprene:methyl farnesoate combinations (Figs. 4C,D). In addition, the independent joint action model predicted a response surface that was less steep relative to the concentration addition model with greater differences predicted between the two models (Figs. 4C,D). Contour lines, generated from the independent joint action model, had virtually no slope at the lower methoprene concentrations. This implies that the lower methoprene

concentrations, within the range evaluated, would have a minimal effect on male sex determination.

The incidence of male progeny production from actual binary combinations of the chemicals were then experimentally determined and compared with the two models of concentration addition and independent joint action. The expected (model) and measured (experimental) responses are presented in Tables 1 and 2. For both binary mixtures the experimental results correlated better to the concentration additive model. Coefficients of determination (r^2) between observed and modeled results according to concentration additivity were 0.69 (pyriproxyfen/methyl farnesoate) and 0.60 (methoprene/methyl farnesoate). Lower correlation coefficients were derived between observed and modeled results when using the independent joint action model (0.09 (pyriproxyfen/methyl farnesoate) and 0.14 (methoprene/methyl farnesoate)). Residuals, the measured minus the expected responses, were consistently lower when using the concentration additive model for all binary combinations used. Residuals also were typically greater than zero in both experiments. These results are consistent with the hypothesis that the JHAs alter sex ratios of offspring by the same mechanism as methyl farnesoate, however, some synergy exists between the JHAs and methyl farnesoate.

DISCUSSION

Having previously established that the juvenoid hormone methyl farnesoate is a male sex determinant in daphnids (Olmstead 2002), we hypothesized that insecticidal JHAs also would influence the sex of offspring through a mechanism of methyl farnesoate agonism. We previously reported that exposure of maternal daphnids to the JHA methoprene altered sex ratios of offspring in favor of males. (Olmstead and LeBlanc 2001). These results are consistent with our current hypothesis. However, methoprene also may have elicited general stress upon the organisms which may stimulate male sex determination among offspring. To test this possibility, maternal daphnids were exposed to several diverse chemicals and effects on offspring sex ratio was determined. Only the juvenoid hormone methyl farnesoate and the JHA pyriproxifen increased the percentage of male offspring born among exposed maternal daphnids. These experiments confirmed that the increased production of male progeny is not a generalized stress response of the daphnids and suggests that insecticidal JHAs function as methyl farnesoate agonists.

Consistent with previous observations with methyl farnesoate, both insecticidal JHAs caused male sex determination during ovarian oocyte maturation. In aphids, juvenile hormone causes loss of one of the sex chromosomes during oocyte maturation (Hales and Mittler 1987). The resulting XO embryos develop into males. We suggest that a similar mechanism of sex determination is operative in daphnids where exposure to methyl farnesoate or JHA insecticides cause sex chromosome diminution to the male genotype. This rather unique period of susceptibility (oocyte maturation) that is common to both the

juvenoid hormone methyl farnesoate and the insecticidal JHAs further supports the hypothesis that the JHAs function as methyl farnesoate agonists in this crustacean species.

Peterson et al. (Peterson 2001) reported that methoprene reduced the production of male progeny in *Daphnia pulex* following a six day exposure of adults. The reason for the discrepancy between this study and our results is not known. Perhaps, experimental conditions favored the action of methoprene as a methyl farnesoate agonist in our studies but favored its action as an antagonist in the Peterson study. The herbicide atrazine was previously reported to stimulate male production by *Daphnia pulicaria* (Dodson, et al. 1999). We were unable to demonstrate the stimulation of male progeny production by atrazine in the present study. Differences in toxicity of this herbicide to the different algal species used as daphnid food in the two studies could be responsible for these discordant results.

Binary combinations of either JHA with methyl farnesoate stimulated male progeny production in a manner that better correlated to the model for concentration additivity than independent joint action. However, both models were deficient in defining the interactions since a synergistic response was evident between the JHAs and the juvenoid hormone. The mechanism responsible for this synergy is not known. A likely scenario involves the ability of the JHAs to interfere with metabolism or clearance of the hormone by competitively binding to enzymes or active transporters that modulate activity or levels of the hormone. Similar synergistic interactions have been reported (Bigley 1979, Pratt 1975, El-Guindy 1980) and further research is required to illuminate the mechanisms behind this combined

response. While neither model precisely defined the combined action of the JHAs with methyl farnesoate, the greater concordance with the model for concentration additivity adds further support that the JHAs stimulate male sex determine by acting as methyl farnesoate agonists.

Trayler and Davis (Trayler 1996) reported a 48-hour LC₅₀ for pyriproxyfen and *Daphnia carinata* of 250 nM. They also noted that exposure of daphnids to 31 nM pyriproxyfen for 14 days significantly reduced fecundity and stimulated resting egg production. Resting, or diapause, eggs are unfertilized haploid eggs and typically are produced following the production of males by females who have entered the sexual reproductive cycle. Thus, it is likely that pyriproxyfen stimulated increased male offspring production in this experiment; however, sex of the offspring were not evaluated. Reduced fecundity (i.e. parthenogenic production of offspring) was likely a consequence of entry of the organisms into the sexual reproductive phase. Schaefer et al. (1990) similarly reported a reduction in fecundity of mixed populations of cladocerans and ostracods at pyriproxyfen exposure levels of 31 nM (Schaefer 1990).

Methoprene's ability to alter sex ratios in some crustacean populations would be of limited toxicological concern under recommended usage conditions as discussed previously (Olmstead and LeBlanc 2001). Male sex determination occurred during acute exposure only at methoprene concentrations that were lethal to some portion of the population. These exposure levels are not likely to be of environmental relevance. However, methoprene also was demonstrated to stimulate male production under experimental conditions and exposure

levels that were not lethal to the organisms (Figs. 2, 4; Olmstead and LeBlanc, 2002). Thus, the sex determining effect of methoprene was not an artifact of differential toxicity to male and female offspring.

The potency with which pyriproxyfen stimulates the production of male offspring may be of concern. Pyriproxyfen has been used historically in the United States for flea and tick control in veterinary applications, and in fire ant bait (Center of Intergrated Pest Management 2002). However, this insecticide is increasingly recommended for agricultural uses such as the control of white fly on cotton and scale insects on fruit trees (Center of Intergrated Pest Management 2002, U.S. Department of Agriculture 2002). While not recommended for direct application to the aquatic environment, like methoprene, the possibility of run-off and leaching into aquatic systems exists where the biological activity of this compound can remain up to 2 months depending upon the amount of organic material in the water (Schaefer, 1988). The extreme potency (100-1000 X that of methyl farnesoate) of this compound and its ability to elicit effects after acute exposure warrants concern in its ability to alter sex ratios in some crustacean populations.

The production of male offspring is the first event occurring when a *Daphnia magna* population switches from parthenogenic to sexual reproduction. The timing of this event needs to be synchronized with alterations in the environment in order to maintain the population's survival over long periods of time. Male production also needs to be timed such that male offspring will be mature and able to mate with females producing resting eggs. Induction of male production by xenobiotics at the wrong time results in mature males that

have no reproductive success due to the absence of resting-egg producing females. These male offspring are then a waste of reproductive output and will result in decreased population growth in subsequent generations.

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FIGURE LEGENDS

Figure 1. Chemical structures of endogenous and synthetic terpenoid hormones.

Juvenile Hormone III and methyl farnesoate are endogenous to insects and crustaceans, respectively. Methoprene and pyriproxyfen are pesticides that function as Juvenile Hormone III mimics.

Figure 2. Effects of various chemicals on male progeny production in daphnid

populations. Bars represent the average and standard deviation of nine individually evaluated daphnid populations. Asterisks indicate a significant difference from the control populations (ANOVA, Dunnett's t-test, $p \leq 0.05$).

Figure 3. Concentration-response curves for the induction of male progeny by methyl

farnesoate, pyriproxyfen, and methoprene. Data were fitted with Equation 1. Each data point represents the average percentage males in individual broods of offspring produced by 5 or 10 maternal daphnids.

Figure 4. Contour plots of the concentration-response surfaces for binary combinations

of pyriproxyfen and methyl farnesoate or methoprene and methyl farnesoate. A)

Concentration additive model for a binary mixture of pyriproxyfen and methyl farnesoate.

B) Independent joint action model for the mixture in 'A'. C) Concentration additive model

for a methyl farnesoate and methoprene mixture. D) Independent joint model for the

mixture in 'C'. Each color along the contour plot represents a 10% increase in the incidence of male progeny per brood.

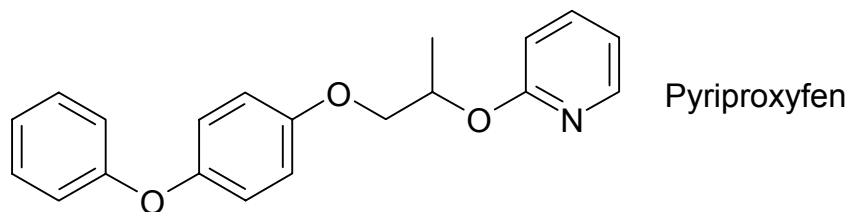
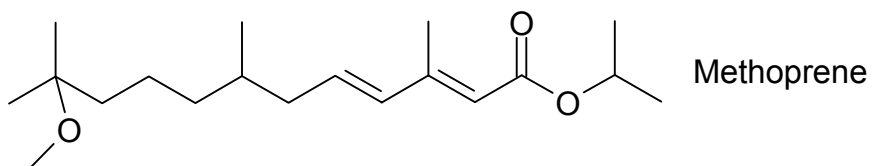
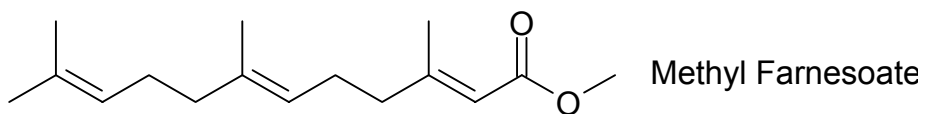
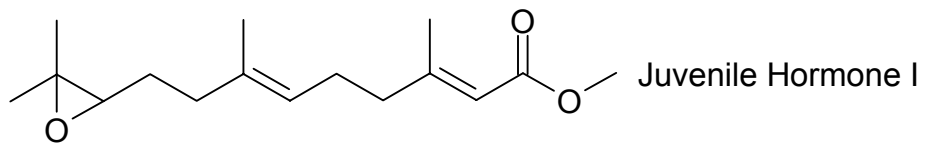


Figure 1

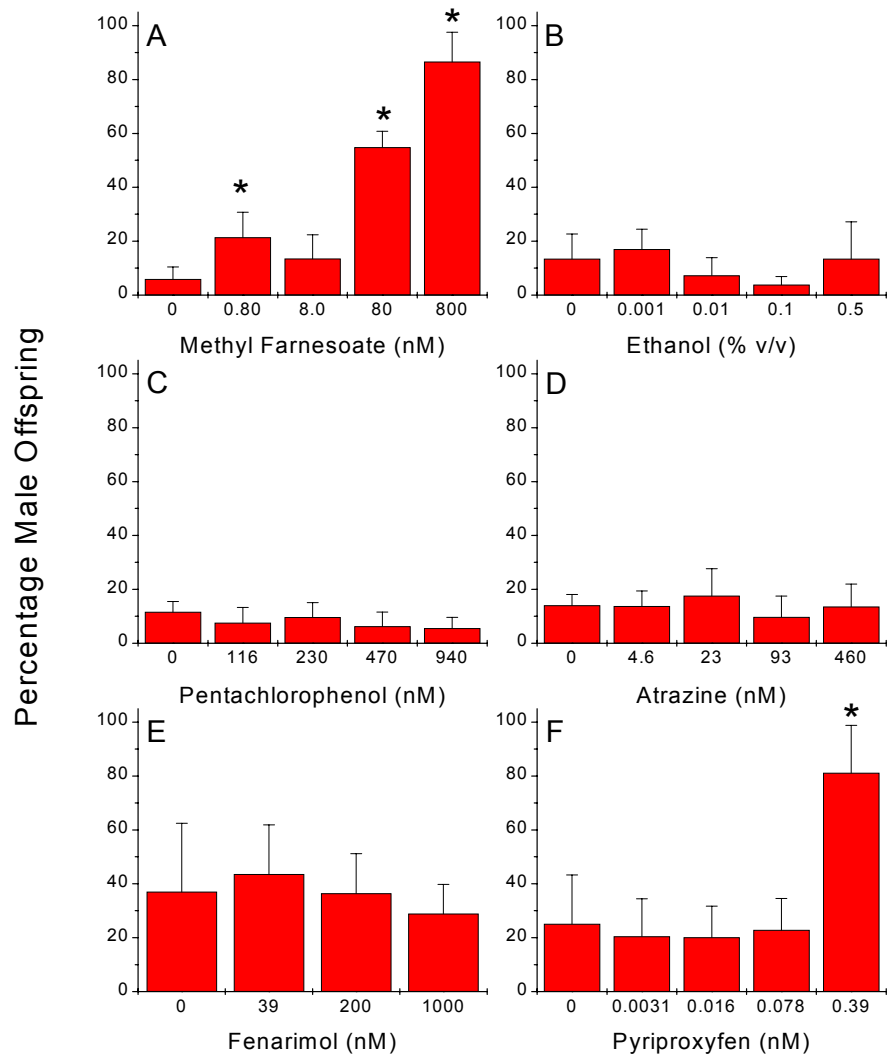


Figure 2

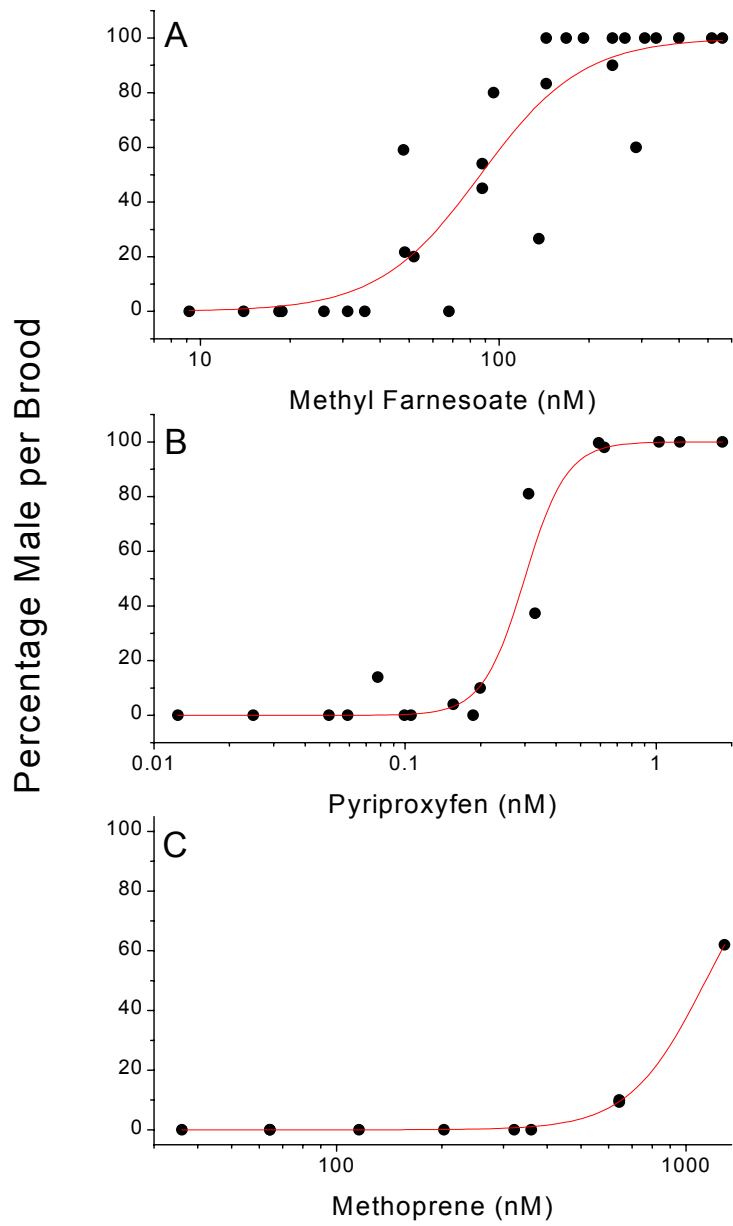


Figure 3

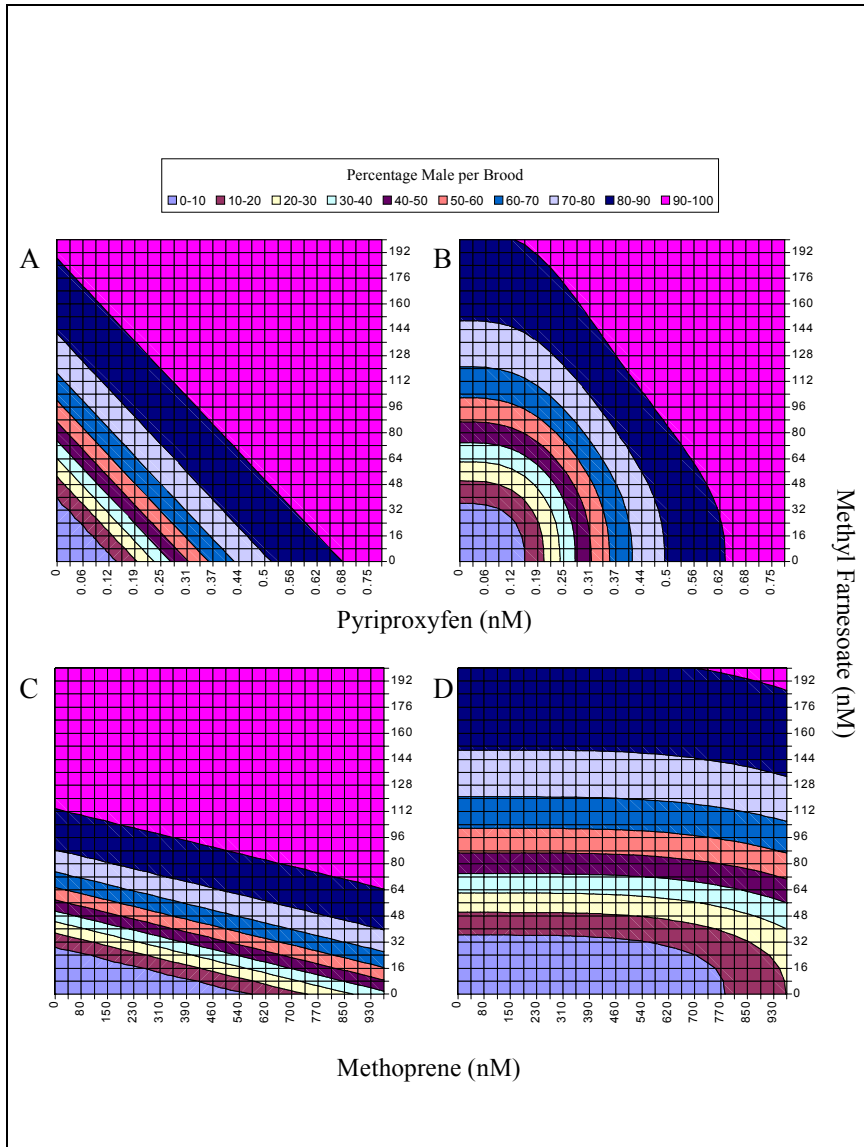


Figure 4

Table 1. Stimulation of the production of male progeny by binary combinations of pyriproxifen and methyl farnesoate and model predictions of the combined exposures.

Observed results (Obs) are presented along with model predictions for concentration addition (CA) and independent joint action (IJA). Data are presented as percentage males per brood. Each experimental data point (Obs) represents the average of 5 individual daphnids. Model predictions were derived from the concentration-response surface models described in Fig. 4.

Pyriproxifen (nM)	Methyl farnesoate (nM)														
	18			36			68			136			264		
	OBS	CA	IJA	OBS	CA	IJA	OBS	CA	IJA	OBS	CA	IJA	OBS	CA	IJA
0.11	15	15	5	12	30	12	86	58	37	89	86	77	100	97	95
0.19	49	35	18	71	50	24	98	71	46	99	90	80	100	97	95
0.33	100	66	56	100	75	58	100	85	70	100	94	89	100	98	97
0.59	100	89	88	100	91	89	100	94	92	100	97	97	100	99	99
1.04	100	97	98	100	98	98	100	98	98	100	99	99	100	99	100

Table 2. Stimulation of the production of male progeny by binary combinations of methoprene and methyl farnesoate and model predictions of the combined exposures.

Observed results (Obs) are presented along with model predictions for concentration addition (CA) and independent joint action (IJA). Data are presented as percentage males per brood. Each experimental data point (Obs) represents the average of 5 individual daphnids. Model predictions were derived from the concentration-response surface models described in Fig. 4.

Methoprene (nM)	Methyl farnesoate (nM)														
	26			48			88			170			310		
	OBS	CA	IJA	OBS	CA	IJA	OBS	CA	IJA	OBS	CA	IJA	OBS	CA	IJA
64	0	3	5	28	17	18	97	56	51	100	90	84	100	98	96
116	0	5	5	13	21	18	92	59	51	100	91	84	100	99	96
200	16	8	5	53	27	19	97	64	51	100	92	84	100	99	96
360	17	17	5	100	39	19	80	72	51	100	93	84	100	99	96
640	91	38	14	100	59	26	100	82	56	100	95	86	100	99	97

SUMMARY

The hypothesis in this research was that daphnid sexual reproduction could be impaired by xenobiotic exposure through a mechanism of endocrine disruption. First, the susceptibility of parameters specific to sexual reproduction to chemical insult was evaluated. Exposure of juvenile daphnids to endocrine-active chemicals altered the development of secondary sex characteristics. Female daphnids exposed to the estrogen, diethylstilbesterol and the juvenile hormone mimic, methoprene had increased development of the abdominal process, a female-specific characteristic. The androgen, androstenedione stimulated the growth of the primary antenna in male daphnids, a male-specific characteristic. Exposure of populations of daphnids to methoprene resulted in an increased production of males as well as a shift in reproductive output from resting egg to neonatal production. These results prove that daphnid sexual reproduction can be targeted by toxic insults and helped lay the foundation for studying underlying mechanisms of these effects.

Mechanistic studies focused on male progeny production and juvenile hormone mimics. The toxicity of methoprene to daphnids was characterized using a variety of endpoints, including fecundity, juvenile growth, molting, and reproductive age from which two distinct mechanisms of action were discerned. The mechanism acting at the lower concentrations specifically affected fecundity and molting and may be indicative of methyl farnesoate disruption. Due to the nature of methoprene's action in insects and the role of juvenile hormones in male production in aphids, the role of methyl farnesoate, the only known crustacean juvenile hormone, in daphnid sex determination was studied. Exposure to methyl farnesoate dramatically increased male production in daphnid populations exposed

chronically to this hormone and at high concentrations resulted in an almost complete shift in sex ratio to all males. This effect on sex determination was found to be the result of exposure during a critical period in oocyte development. Complete sex reversal was observed in daphnid offspring exposed to methyl farnesoate for a 24 hour window during ovarian maturation. Further, this effect was only observed when the exposure occurred within the 24 hours before the offspring were released from the ovary into the brood chamber. These experiments defined the role of methyl farnesoate in daphnid sex determination and provided a mechanism of action, namely methyl farnesoate disruption, for the induction of male offspring observed in daphnids exposed to methoprene.

Further experiments were performed to determine the specificity of daphnid's response to juvenile hormone mimics and support a mechanism of methyl farnesoate disruption for these chemicals. Pyriproxyfen, another juvenile hormone mimicking insecticide also induced the production of males in chronically exposed populations of daphnids. Other chemicals of varying toxicant action were not able to alter sex ratios in exposed populations, suggesting that this skewing of sex ratios in favor of males is a relatively unique characteristic of juvenile hormone mimicking compounds. Pyriproxyfen and methoprene also altered sex ratios of offspring after acute exposure during the critical window for sex determination as observed with methyl farnesoate. Binary mixtures methyl farnesoate:pyriproxyfen and methyl farnesoate:methoprene stimulated the production of male offspring in a manner that more closely fit a model of concentration addition than an independent joint action model suggesting that these insecticides affect the same physiological target as methyl farnesoate. This research demonstrated that xenobiotics are in

fact capable of altering sexual reproduction in daphnids, that exposure to juvenile hormone mimics results in methyl farnesoate disruption in crustaceans, and that sex determination in daphnids can be used as a tool for investigating the disruption of methyl farnesoate-regulated processes.

DISCUSSION

As cyclical parthenogens, *Daphnia magna* alternate between two reproductive modes; parthenogenesis (asexual) when conditions are favorable and sexual reproduction in order to prepare for inhospitable conditions (Moss, 1998) (Pennak, 1953). For example, in the spring, food resources are abundant as algae density increases and general environmental conditions favor daphnid survival (Moss, 1998). During this time *Daphnia magna* reproduce by parthenogenesis (asexual reproduction), allowing them to clonally expand and exploit these favorable growing conditions. In late summer and fall as these conditions deteriorate with the coming of winter, daphnids will switch to sexual reproduction, and produce dormant resting eggs which accumulate as egg banks on the bottom of the habitat (Caceres, 1998; Frey, 1982). During the harsher conditions of winter when adult survival is limited or nonexistent, these resting eggs will remain dormant in the sediment until spring when they resume development, hatch as juvenile females, and then re-establish the adult population in a given location (Moss, 1998).

Alterations in sexual reproduction due to xenobiotic exposure could impair the long term health of daphnid populations. The presence of viable egg banks at the bottom of lakes and ponds ensures the ability of these daphnids to establish populations over the successive years (Moss, 1998). The resting eggs contained within these ephippia can remain viable for over 20 years (Caceres, 1998) and thus maintain a daphnid population over long successive periods of environmental adversity. A reduction in the accumulation of viable resting eggs would slowly diminish the ability of these egg banks to re-establish the active population and increase the chance of local extinction.

Methoprene stimulated male production in exposed populations and shifted reproductive output from resting egg production to neonatal production. Overall this could have a doubly negative impact on daphnid sexual reproduction. It is important that the production of male progeny and resting eggs be timed such that, when the males are mature, there are female daphnids producing resting eggs that the males can fertilize. Producing male offspring too early results in males that mature but cannot reproduce due to the lack of females producing resting eggs to be fertilized. These males are unable to reproduce and are essentially wasted reproductive output. This effect is further enhanced by methoprene's ability to shift reproductive output away from resting egg production to neonatal production. That methoprene had incongruous effects on male production (increase) and the production of resting eggs (decrease) suggest that these two events in sexual reproduction are controlled separately from one another even though they occur sequentially in natural populations.

The environmental cues that trigger sexual reproduction have been studied extensively (Deng, 1996; Kleiven *et al.*, 1992; Korpelainen, 1989; Stross and Hill, 1965); however, the physiological responses that result in the shift from parthenogenesis to sexual reproduction are unknown. Exposure to the terpenoid hormone, methyl farnesoate, resulted in increased production of male offspring in a similar fashion to methoprene (Olmstead and LeBlanc, 2002). This effect was found to be due to acute exposure during a specific window of development, when the affected oocyte was maturing in the ovary. Exposure outside of this window had no effect on the sex of the progeny (i.e., all developed into females) while exposure to sufficiently high methyl farnesoate concentrations (> 30 nM) during this window

resulted in all exposed oocytes developing into males (Olmstead and LeBlanc, 2002). Based upon this research, a model for the switch from female to male progeny production can be generated. Environmental stress such as crowding and reduced food availability results in increased methyl farnesoate levels. When oocytes are maturing in the ovaries these increased methyl farnesoate levels signal transcriptional events that result in these oocytes differentiating into males upon further development.

In insects, juvenile hormones exert their effects by acting on a nuclear receptor called ultraspiracle (Jones and Sharp, 1997). A similar receptor for methyl farnesoate action in crustaceans has been discovered (Chang *et al.*, 1998). Activation of this receptor by juvenile hormone analogs would explain the increased male production that occurs when daphnids are exposed to either methoprene or pyriproxyfen (Olmstead and LeBlanc, 2003) and could explain effects observed in other studies. During the methoprene and pyriproxyfen experiments it was noted that exposed daphnids became red in color compared to control daphnids. This color difference was due to increased concentrations of hemoglobin due to induction of the hemoglobin 2 gene (LeBlanc, unpublished). Experiments with gel mobility shift assays, revealed binding activity in the promoter region of the hemoglobin 2 gene in pyriproxyfen and methyl farnesoate exposed daphnids. This binding domain appears to be a nuclear receptor binding half-site. Binding to this region is more pronounced in pyriproxyfen-exposed daphnids and as such follows the same trend of greater potency of the insecticide in male determination assays when compared to the hormone (LeBlanc and Gore, unpublished). This work supports the hypothesis that pyriproxyfen and similar juvenile hormone analogs interact with a nuclear methyl farnesoate receptor in daphnids.

The presence of methyl farnesoate as a juvenile hormone in crustaceans was only recognized fairly recently (Laufer *et al.*, 1987) and its role in crustacean endocrinology is not well characterized (Homola and Chang, 1997; Laufer and Biggers, 2001). Methyl farnesoate appears to have functions similar to the juvenile hormones of insects with regard to juvenile growth and development. For example application of methyl farnesoate retarded the metamorphosis of late larval lobsters (*Homarus americanus*) (Borst *et al.*, 1987) and caused the development of intermediate stages in late larval prawns (*Macrobrachium rosenbergii*) (Abdu *et al.*, 1998). The opposite effect was observed in the larva of barnacles (*Balanus amphitrite*), however, where methyl farnesoate exposure induced metamorphosis (Yamamoto *et al.*, 1997). Methyl farnesoate has also been suggested to play a role in crustacean reproduction. Hemolymph titers of this hormone were found to increase naturally with ovarian maturation in spider crabs (*Libinia emarginata*) (Laufer *et al.*, 1987) and have been correlated to male reproductive morphology in the same species (Homola *et al.*, 1991; Sagi *et al.*, 1993). Treatment with methyl farnesoate causes increased reproductive output in shrimp (*Penaeus vannamei*) (Laufer *et al.*, 1997) and stimulates ovarian maturation and growth in crayfish (*Procambarus clarkii*) (Laufer *et al.*, 1998) and crabs (*Oziotelphusa senex*) (Reddy and Ramamurthi, 1998). Understanding the role methyl farnesoate appears to play in daphnid sex determination improves this growing base of knowledge on the functions of this hormone in crustaceans. Given its effects in daphnids and titer differences in spider crab male morphotypes, methyl farnesoate may play important roles in establishing and maintaining sexual characteristics in crustaceans.

Certain biorational pesticides are designed to target juvenile hormone-regulated processes in pest insect species (Dhadialla *et al.*, 1998; Retnakaran *et al.*, 1985). Since juvenile hormones are unique to arthropod species, these chemicals exhibit relatively little toxicity to non-arthropod species. These insecticides do, however, exhibit high toxicity to some crustacean species (Chu *et al.*, 1997; Gomez *et al.*, 1973; McKenney and Celestial, 1996; Olmstead and LeBlanc, 2001; Trayler and Davis, 1996). Based on the similarities between insect and crustacean endocrinologies as well as the results reported here, it is likely that in crustaceans these juvenile hormone analogs are acting as endocrine disruptors, interfering with methyl farnesoate-regulated processes. Linking the effects of juvenile hormone-mimicking toxicants on crustaceans to a mechanism of methyl farnesoate disruption yields a better understanding of and the ability to detect endocrine disruption in crustacean species. Exposure of crustaceans to these types of chemicals have resulted in delayed larval development (Celestial and McKenney, 1994; Chu *et al.*, 1997; McKenney and Matthews, 1990; Templeton and Laufer, 1983) and impaired reproduction (Chu *et al.*, 1997; McKenney and Celestial, 1996; Payen and Costlow, 1977; Templeton and Laufer, 1983). These effects are poor indicators of methyl farnesoate disruption because numerous other chemicals with differing modes of actions can cause these same effects. Daphnid sex determination, though, appears to be uniquely affected by juvenile hormone mimics and may be useful as a specific endpoint of methyl farnesoate disruption.

This research comprises a new model for the study of endocrine disruption in crustaceans. It provides a very specific endpoint, sexual differentiation, for investigating the effects of xenobiotic exposure on the methyl farnesoate hormonal axis. The economic and

ecological value of crustaceans are such that possible endocrine disruption in this group of organisms needs to be studied and understood. These juvenile hormone-mimicking pesticides are used in a variety of media including veterinary, agricultural, and mosquito control applications (Dhadialla *et al.*, 1998; Retnakaran *et al.*, 1985). In the case of mosquito control, these compounds are especially environmentally relevant as they are applied directly to the aquatic environment and can be detected in appreciable amounts (Knuth, 1989; Ross *et al.*, 1994; Schaefer *et al.*, 1988). This work provides a model for studying methyl farnesoate disruption in a crustacean species as well as a rapid means of identifying methyl farnesoate mimics in acute male production experiments.

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