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

Allen W. Olmstead and Gerald A. LeBlanc

Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, North Carolina, USA

Juvenile hormone analogs (JHAs) represent a class of insecticides that were designed specifically 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, producing altered sex ratios of offspring. Daphnids were exposed chronically (3 weeks) to sublethal concentrations of methyl farnesoate, the JHA pyriproxyfen, and several nonjuvenoid chemicals 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 hr) to either pyriproxyfen or the JHA methoprene caused oocytes maturing in the ovary to develop into males. We performed experiments to determine whether combined effects of a JHA and methyl farnesoate conformed better 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, although 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. *Key words:* **crustacea, *Daphnia*, endocrine disruption, invertebrate, methoprene, methyl farnesoate, mixtures, pyriproxyfen.** *Environ Health Perspect* **111:919–924 (2003). doi:10.1289/ehp.5982 available via *http://dx.doi.org/* [Online 5 February 2003]**

Environmental sex determination occurs in a species when the sex of offspring is dictated 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 maximize 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 generates 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).

Although 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 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 and LeBlanc 2002). Thus, methyl farnesoate is a likely endocrine factor that transduces the environmental cues (changes in photoperiod, reduced food) to the physiologic response (production of male offspring). Methyl farnesoate is a terpenoid hormone synthesized by the mandibular organ in decapod crustaceans (Borst et al. 1994; Ding and Tobe 1991) and is involved in various aspects of crustacean reproduction and juvenile development (Homola and Chang 1997; Laufer and Biggers 2001; Laufer et al. 1993). We propose that methyl farnesoate stimulates male progeny production by activating an ultraspiracle-like receptor in daphnids. Ultraspiracle is a retinoid X receptor ortholog found in insects and crustaceans (Chung et al. 1998; Oro et al. 1990). Methyl farnesoate is structurally very similar to the insect terpenoid juvenile hormone III (Figure 1), which can activate ultraspiracle (Jones and Sharp 1997).

In a previous study, we reported that chronic exposure of daphnids to the insecticidal juvenile hormone analog (JHA) methoprene shifted sex ratios of offspring toward males compared with 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 the stimulation of male production was unique to JHAs. We also evaluated the ability of the JHAs methoprene and pyriproxyfen to program sex in oocytes during 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 (Safe 1990). 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.

Materials and Methods

Daphnid culture. Daphnids (*Daphnia magna*) were cultured in incubators at a density of 40 adults in 1 L of medium at a temperature and photoperiod of 20°C and 16 hr light. Algae (*Selenastrum capricornutum*), cultured in Bold's basal medium, was used as a food source for daphnids during culturing and

Address correspondence to G.A. LeBlanc, Department of Environmental and Molecular Toxicology, 850 Main Campus Dr., North Carolina State University, Raleigh, NC 27695-7633 USA. Telephone: (919) 515-7404. Fax: (919) 515-7169. E-mail: GA_LeBlanc@ncsu.edu

This work was supported by U.S. Environmental Protection Agency STAR Program Grants R82612901 and R82935801 to G.A.L.

The authors declare they have no conflict of interest.

Received 6 September 2002; accepted 5 February 2003.

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 determined previously that the strain of daphnids used in our laboratory produces male progeny 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 JHA 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 hr 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 20°C under a 16-hr light photoperiod. Solutions were changed and offspring removed every 3 days. Sex of individual offspring was determined microscopically (10× 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 affected parthenogenic reproduction in standard life cycle tests. The pesticidal JHA pyriproxyfen (Chem Service, West Chester, PA) 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 M. Feldlaufer, U.S. Department of Agriculture, Beltsville, MD; provided by H.H. Rees and G. Wainwright, University of Liverpool, Liverpool, UK) was used as the positive control (Olmstead and LeBlanc 2002). The herbicide atrazine (Chem Service) was evaluated because this chemical was reported previously to stimulate male progeny production (Dodson et al. 1999). Fenarimol (Chem Service) was selected because this fungicide functions as an antiecdysteroid (Mu and LeBlanc 2002), and ecdysteroids have been implicated in male progeny production (Peterson et al. 2001). Pentachlorophenol (Chem Service), a polar narcotic and an uncoupler of oxidative phosphorylation (Schuurmann et al. 1997), was used to determine whether male progeny production occurs in response to general metabolic stress. Atrazine, fenarimol, and pentachlorophenol were selected for use in these experiments because they all had the potential to stimulate male offspring production although they are nonjuvenoid in structure and function. Ethanol (Aaper, Shelbyville, KY) 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.

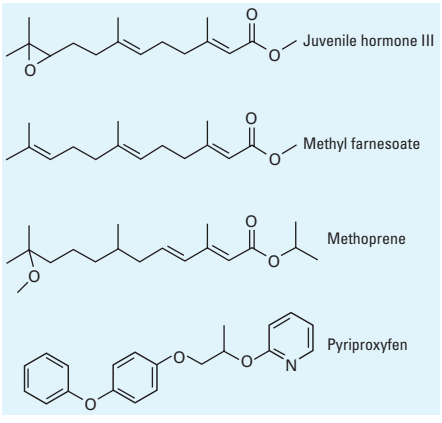

Figure 1. Chemical structures of endogenous and synthetic terpenoid hormones. Juvenile hormone III and methyl farnesoate are endogenous to insects and crustaceans, respectively. Methoprene and pyriproxyfen are pesticides that function as juvenile hormone III mimics.

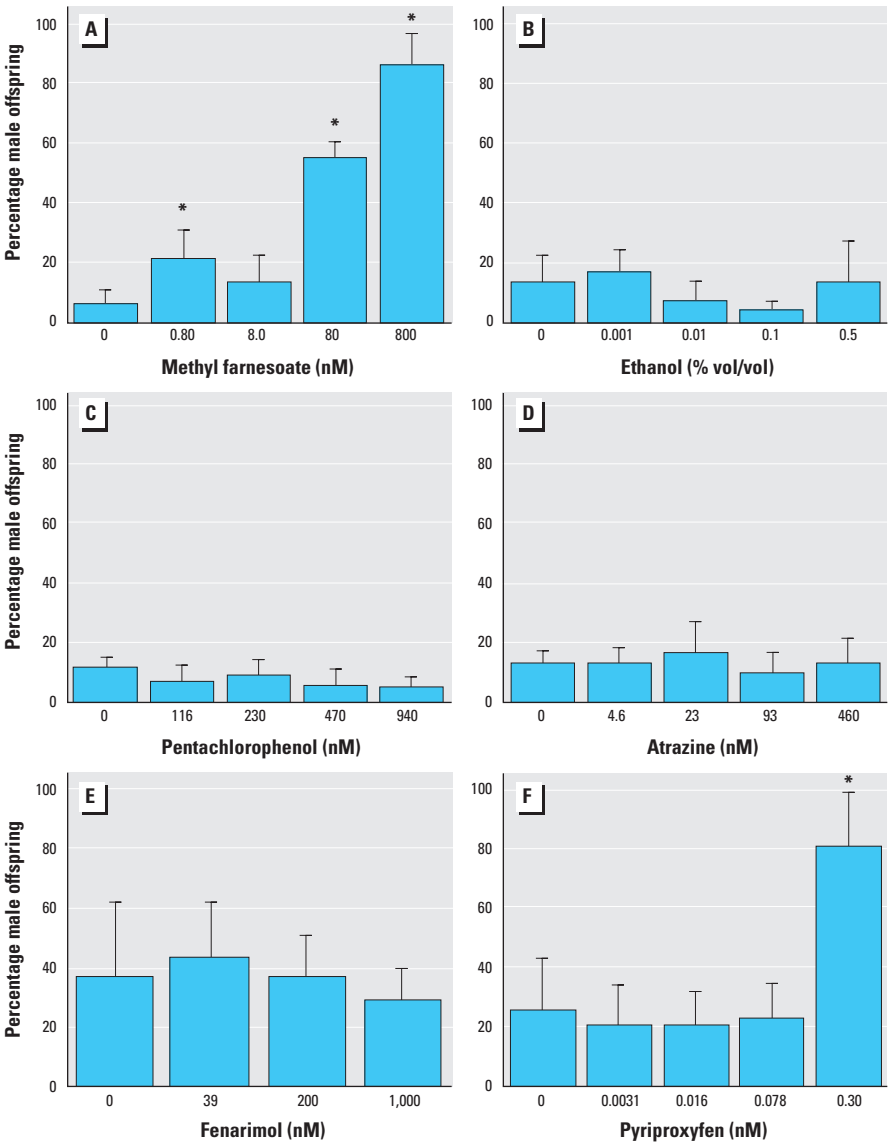

Figure 2. Effects of various chemicals on male progeny production in daphnid populations. Bars represent the average and standard deviation (error bars) of nine individually evaluated daphnid populations.
*Significantly different from the control populations (ANOVA, Dunnett's *t*-test, $p \leq 0.05$).

The concentration of carrier present in any given test solution never exceeded 0.005% vol/vol. 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 analysis of variance (ANOVA) and Dunnett's *t*-test (JMP software; SAS Institute, Cary, NC).

Exposure to JHAs during oocyte development. Methyl farnesoate was previously shown to program maturing oocytes in the ovary to develop into males (Olmstead and LeBlanc 2002). After 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 hr for the presence of a molted exoskeleton. Forty-eight hours after detecting a molted exoskeleton, we transferred the daphnid to test media containing the appropriate concentration of the test chemical. The daphnid was maintained in this solution for 24 hr, 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 cells, and fish food homogenate, 0.2 mg dry weight) was provided to each beaker twice daily. Daphnids typically produce only female offspring under these nonstressed 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) using the following concentration–response equation:

$$R = \frac{100}{1+10^{\left[\log(EC_{50})-\log(C_x)\right]p}} \qquad [1]$$

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, which is the percentage of males per brood. EC_{50} values for each chemical were determined from these fitted equations. The EC_{50} was the calculated concentration of the material that caused a 50% incidence of male offspring.

Binary combinations. A zero-interaction concentration–response surface for the concentration-additive model was generated using the fitted concentration–response curves determined 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; Pöch 1993). The equation used to generate this curve was derived from Equation 1 by adjusting the second chemical's concentration (C_y) to equivalencies of the first chemical (C_x) using a relative potency factor (EC_{50x}/EC_{50y}):

$$R_c = \frac{100}{1+10^{\log EC_{50x}-\log\left(C_x+C_y\times\frac{EC_{50x}}{EC_{50y}}\right)p'}} \qquad [2]$$

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:

$$R_c = R_x + R_y - R_xR_y \qquad [3]$$

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 described above. 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 with actual results by calculation of coefficients of determination (r^2) for each model (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 (Figure 2). Under these exposure conditions, pyriproxyfen was 2–3 orders of magnitude more potent at stimulating male progeny production than was methyl farnesoate. Altered sex ratios were not caused by differential embryo mortality because 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, but appears to be specific to juvenoid hormones.

Male-sex determination during oocyte exposure. We performed experiments 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 was determined. Exposure of oocytes to methyl farnesoate during ovarian development programmed the oocytes to develop into male offspring in a

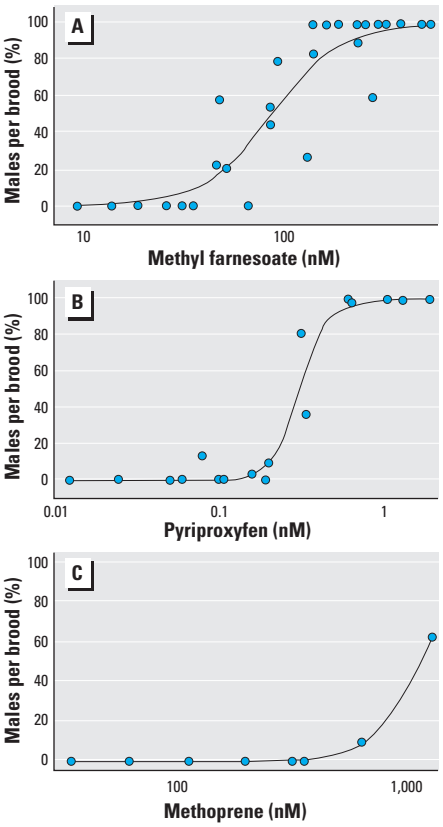

Figure 3. Concentration–response curves for the induction of male progeny by methyl farnesoate, pyriproxyfen, and methoprene. Data were fitted with Equation 1. Each data point represents the average percentage males in individual broods of offspring produced by 5 or 10 maternal daphnids.

concentration-dependent manner (Figure 3A) with an EC_{50} of 87 nM. Pyriproxyfen stimulated male progeny production among oocytes during ovarian development (Figure 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 1,140 nM). The EC_{50} value for male production by methoprene was approximately the concentration that is lethal to 50% of exposed neonatal daphnids (LC_{50} = 1,160 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 (Figure 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 (Figure 4) to illustrate differences between the concentration-addition and independent joint-action models. 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 (Figure 4A) and were concave using the independent joint-action model (Figure 4B). The concave character of the contour plot generated with the independent joint-action model indicates that combined effects predicted with this model are less then those predicted by simple concentration additivity.

Similar contrasts between the shape of the response surface were evident with the methoprene–methyl farnesoate combinations (Figure 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 (Figure 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 was 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 r^2 values were derived between observed and modeled results when using the independent joint-action model (0.09, pyriproxyfen–methyl farnesoate; 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.

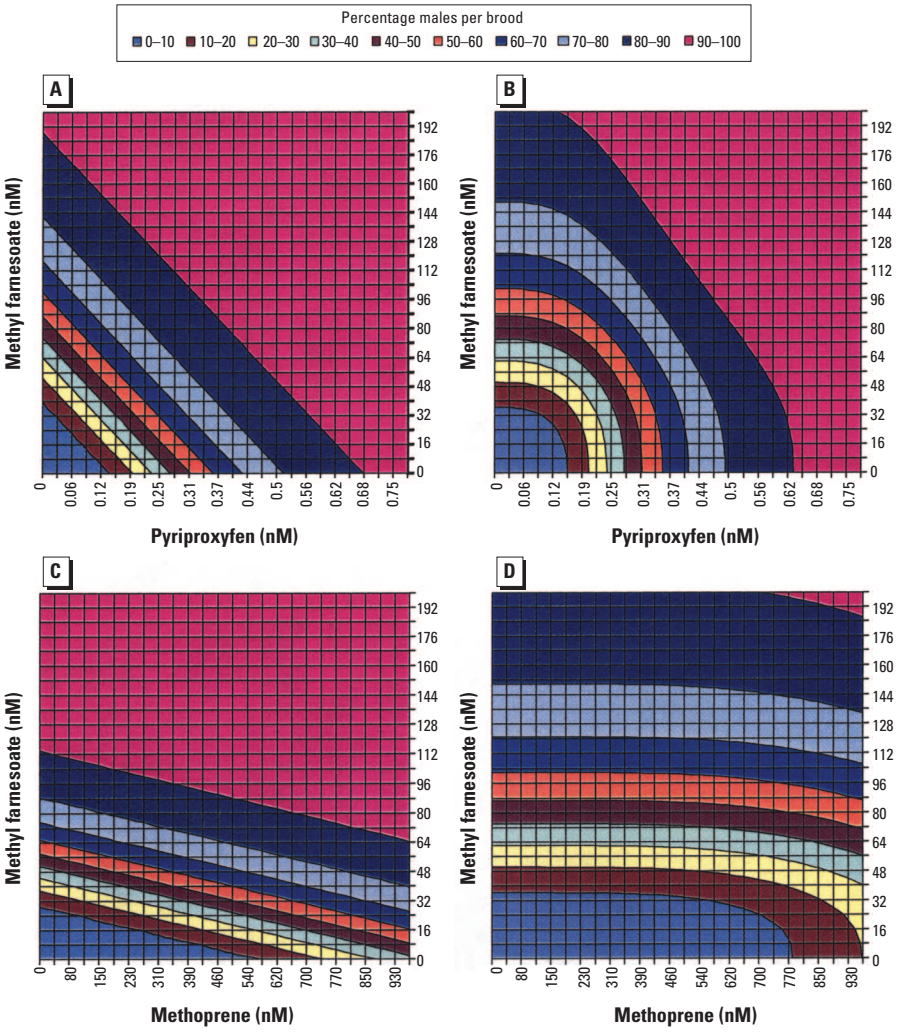

Figure 4. Contour plots of the concentration–response surfaces for binary combinations of pyriproxyfen and methyl farnesoate or methoprene and methyl farnesoate. (*A*) Concentration-additive model for a binary mixture of pyriproxyfen and methyl farnesoate. (*B*) Independent joint-action model for the mixture in (*A*). (*C*) Concentration-additive model for a methyl farnesoate and methoprene mixture. (*D*) Independent joint-action model for the mixture in (*C*). Each color along the contour plot represents a 10% increase in the incidence of male progeny per brood.

Discussion

Having previously established that the juvenoid hormone methyl farnesoate is a male sex determinant in daphnids (Olmstead and LeBlanc 2002), we hypothesized that insecticidal JHAs also would influence the sex of offspring through a mechanism of methyl farnesoate agonism. We reported previously that exposure of maternal daphnids to the JHA methoprene altered sex ratios of offspring in favor of males (Olmstead and LeBlanc 2001). Those 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, we exposed maternal daphnids to several diverse chemicals, and determined the effects on offspring sex ratios. Only the juvenoid hormone methyl farnesoate and the JHA pyriproxyfen 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 suggest 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, the absence of juvenile hormone causes loss of one of the sex chromosomes during oocyte maturation (Hales and Mittler 1987). The resulting X0 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 causes sex chromosome diminution to the male genotype. This 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. (2001) reported that methoprene reduced the production of male progeny in *Daphnia pulex* after a 6-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 Peterson et al.'s (2001) 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 have caused differential food deprivation and resulting differences in male offspring production.

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 with that for independent joint action. However, both models were deficient in defining the interactions because 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 and Vinson 1979; El-Guindy et al. 1980; Pratt 1975), and further research is required to illuminate the mechanisms behind this combined response. Although neither model precisely defined the combined action of the JHAs with methyl farnesoate, the greater concordance with the model for concentration additivity contributes further support that the JHAs stimulate male sex determination by acting as methyl farnesoate agonists.

Trayler and Davis (1996) reported a 48-hr LC_{50} 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 haploid eggs that require fertilization and typically are produced after 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 was not evaluated. Reduced fecundity (i.e., reduced parthenogenic production of offspring) was likely a consequence of entry of the organisms into the sexual reproductive phase. Schaefer and Miura (1990) similarly reported a reduction in fecundity of mixed populations of cladocerans and ostracods at pyriproxyfen exposure levels of 31 nM (Schaefer and Miura 1990).

Methoprene's ability to alter sex ratios in some crustacean populations would be of limited toxicologic 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 (Table 2; Olmstead and LeBlanc 2002). Therefore, 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 veterinarian applications, and in fire ant bait (Center of Integrated 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 Integrated Pest Management 2002). Although pyriproxyfen is not recommended for direct application to the aquatic environment, the possibility of runoff and leaching into aquatic systems exists, and there the biologic activity of this compound can remain up to 2 months depending upon the amount of organic material in the water (Schaefer et al. 1988). The extreme potency (100× to 1,000× that of methyl farnesoate) of pyriproxyfen and its ability to elicit effects after acute exposure warrants concern in its ability to alter sex ratios in some crustacean populations.

Alterations in sex determination could have dire consequences to populations of cyclic parthenogens such as *D. magna*. The production of male offspring is an early event in the transition from parthenogenic to sexual reproduction by these organisms. Sexual reproduction is critical to the survival of populations during periods of environmental adversity (i.e., winter), whereas parthenogenic reproduction allows for high fecundity and rapid population growth during periods of

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

Pyriproxyfen (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

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 five individual daphnids. Model predictions were derived from the concentration–response surface models depicted in Figure 4.

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

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

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 five individual daphnids. Model predictions were derived from the concentration–response surface models depicted in Figure 4.

high resource availability. The aberrant production of males by insecticide exposure could interfere with asexual population growth, threatening sustenance of the population as well as populations of consumers that rely upon the daphnids as an energy source.

References

Baldwin WS, LeBlanc GA. 1994. Identification of multiple steroid hydroxylases in *Daphnia magna* and their modulation by xenobiotics. Environ Toxicol Chem 13:1013–1021.

Bergeron JM, Crews D, McLachlan JA. 1994. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. Environ Health Perspect 102:780–781.

Bigley W, Vinson S. 1979. Effects of piperonyl butoxide and DEF on the metabolism of methoprene by the imported fire ant, *Solenopsis invicta* Buren. Pestic Biochem Physiol 10:14–22.

Bliss C. 1939. The toxicity of poisons applied jointly. Ann Appl Biol 26:585–615.

Borst D, Tsukimura B, Laufer H, Couch E. 1994. Regional differences in methyl farnesoate production by the lobster mandibular organ. Biol Bull 186:9–16.

Center of Integrated Pest Management. 2002. North Carolina Agricultural Chemicals Manual. Raleigh, NC:North Carolina State University. Available: http://ipmwww.ncsu.edu/agchem/agchem.html [accessed 16 August 2002].

Chung ACK, Durica DS, Clifton SW, Roe BA, Hopkins PM. 1998. Cloning of crustacean ecdysteroid receptor and retinoid-X receptor gene homologs and elevation of retinoid-X receptor mRNA by retinoic acid. Mol Cell Endocrinol 139:209–227.

Ding Q, Tobe S. 1991. Production of farnesoic acid and methyl farnesoate by mandibular organs of the crayfish, *Procambarus clarkii*. Insect Biochem 21:285–291.

Dodson S, Merritt C, Shannahan J, Shults C. 1999. Low dose concentrations of atrazine increase male production in *Daphnia pulicaria*. Environ Toxicol Chem 18:1568–1573.

El-Guindy M, El-Sattar M, Madi S. 1980. Synergists as potentiators to juvenile hormone analogues in susceptible and fenitrothion-resistant strains of the cotton leafworm *Spodoptera littoralis* (Boisd). Z Ang Ent 90:520–525.

Gessner P. 1995. Isobolographic analysis of interactions: an update on applications and utility. Toxicology 105:161–179.

Hales DF, Mittler TE. 1987. Chromosomal sex determination in aphids controlled by juvenile hormone. Genome 29:107–109.

Hebert PDN. 1978. The population biology of *Daphnia* (Crustacea, Daphnidae). Biol Rev 53:387–426.

Homola E, Chang ES. 1997. Methyl farnesoate: crustacean juvenile hormone in search of functions. Comp Biochem Physiol B Biochem Mol Biol 117:347–356.

Jones G, Sharp PA. 1997. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. Proc Natl Acad Sci USA 94:13499–13503.

Korpelainen H. 1990. Sex ratios and conditions required for environmental sex determination in animals. Biol Rev 65:147–184.

Laufer H, Ahl JSB, Sagi A. 1993. The role of juvenile hormones in crustacean reproduction. Am Zool 33:365–374.

Laufer H, Biggers W. 2001. Unifying concepts learned from methyl farnesoate for invertebrate reproduction and postembyronic development. Am Zool 41:442–457.

Mu X, LeBlanc GA. 2002. Environmental antiecdysteroids alter embryo development in the crustacean *Daphnia magna*. J Exp Zool 292:287–292.

Olmstead AW, LeBlanc GA. 2000. Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. Environ Toxicol Chem 19:2107–2113.

———. 2001. Temporal and quantitative changes in sexual reproductive cycling of the cladoceran *Daphnia magna* by a juvenile hormone analog. J Exp Zool 290:148–155.

———. 2002. Methyl farnesoate is a sex determinant in *Daphnia magna*. J Exp Zool 293:736–739

Oro A, McKeown M, Evans R. 1990. Relationship between the product of the Drosophila ultraspiracle locus and the vertebrate retinoid X receptor. Nature 347:298–301.

Peterson J, Kashian D, Dodson S. 2001. Methoprene and 20-OH-ecdysone affect male production in *Daphnia pulex*. Environ Toxicol Chem 20:582–588.

Pöch G. 1993. Combined Effects of Drugs and Toxic Agents. New York:Springer.

Pratt G. 1975. Inhibition of juvenile hormone carboxyesterase of locust hemolymph by organophosphates in vitro. Insect Biochem 5:595–607.

Safe S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). CRC Crit Rev Toxicol 21:51–70.

Schaefer C, Miura T. 1990. Chemical persistence and effects of S-31183, 2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine, on aquatic organisms in field tests. J Econ Entomol 83:1768–1776.

Schaefer C, Miura T, Dupras E, Mulligan F, Wilder W. 1988. Efficacy, nontarget effects, and chemical persistence of S-31183, a promising mosquito (Diptera: Culicidae) control agent. J Econ Entomol 81:1648–1655.

Schuurmann G, Segner H, Jung K. 1997. Multivariate mode-of-action analysis of acute toxicity of phenols. Aquat Toxicol 38:277–296.

Trayler K, Davis J. 1996. Sensitivity of *Daphnia carinata* sensu lato to the insect growth regulator, pyriproxyfen. Ecotoxicol Environ Safety 33:154–156.

Wolf HG, Carvalho GR. 1989. Resting eggs of lake *Daphnia*. II. *In situ* observations on the hatching of eggs and their contribution to population and community structure. Freshwater Biol 16:1269–1276.

Zar JH. 1996. Biostatistical Analysis. Upper Saddle River, NJ:Prentice-Hall.