The screening of chemicals for juvenoid-related endocrine activity using the water flea *Daphnia magna*

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Abstract

U.S. Environmental Protection Agency is charged with developing a screening and testing paradigm for detecting endocrine toxicity of chemicals that are subject to regulation under the Food Quality Protection and the Safe Drinking Water Acts. In this study, we developed and evaluated a screening assay that could be employed to detect juvenoid-related endocrine-modulating activity in an invertebrate species. Juvenoid activity, anti-juvenoid activity, and juvenoid potentiator activity of chemicals was assessed using the water flea *Daphnia magna*. Male sex determination is under the regulatory control of juvenoid hormone, presumably methyl farnesoate, and this endpoint was used to detect juvenoid modulating activity of chemicals. Eighteen chemicals were evaluated for juvenoid agonist activity. Positive responses were detected with the juvenoid hormones methyl farnesoate and juvenile hormone III along with the insect growth regulating insecticides pyriproxyfen, fenoxycarb, and methoprene. Weak juvenoid activity also was detected with the cyclodiene insecticide dieldrin. Assays performed repetitively with compounds that gave either strong positive, weak positive, or negative response were 100% consistent indicating that the assay is not prone to false positive or negative responses. Five candidate chemicals were evaluated for anti-juvenoid activity and none registered positive. Four chemicals (all *trans*-retinoic acid, methoprene, kinoprene, bisphenol A) also were evaluated for their ability to potentiate the activity of methyl farnesoate. All registered positive. Results demonstrate that an in vivo assay with a crustacean species customarily employed in toxicity testing can be used to effectively screen chemicals for juvenoid-modulating activity.
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1. Introduction

Under the auspices of the Food Quality Protection and the Safe Drinking Water Acts of 1996, Congress mandated that the U.S. Environmental Protection Agency develop and implement procedures for the screening and testing of chemicals for endocrine toxicity. The Endocrine Disruptors Screening and Testing Committee (EDSTAC) was established in response to this mandate. The role of EDSTAC was to recommend a screening and testing paradigm to EPA that would be both scientifically sound and

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meet the concerns of stakeholders. A two-tier system was proposed (EDSTAC, 1998), whereby chemicals would be screened for evidence of estrogen, androgen, and thyroid (EAT)-related activities under Tier 1. Chemicals identified as being endocrine active under Tier 1 screening then would be tested under Tier 2 with the intent of definitively establishing a no observed effect level (NOEL) for the compound. The proposed Tier 1 screening and Tier 2 testing utilize a diverse set of species in order to ensure that species differences in sensitivity and susceptibility to endocrine toxicants are considered.

Among the species recommended for testing by EDSTAC was one invertebrate—the mysid shrimp. However, this species was recommended for use only in the Tier 2 testing. Therefore, Tier 2 testing with the mysid shrimp would be justified by screening tests performed with vertebrate species. Invertebrates possess EAT-like activities though the hormones involved differ significantly from those of vertebrates. Accordingly, susceptibility of invertebrate EAT-like activities to endocrine toxicants would likely differ significantly from those of vertebrates.

In arthropods (crustaceans, insects, and some lesser phyla), EAT-like activities are mediated largely through the action of two hormone/receptor types: juvenoids and ecdysteroids (LeBlanc et al., 1999). The juvenoids are structurally similar to the retinoids of vertebrates (Nijhout, 1998), while the ecdysteroids share, in some respects, similarity with vertebrate estrogens and androgens (De Loof and Huybrechts, 1998). In the absence of a Tier 1 screening assay with an invertebrate, all chemicals tested in Tier 2 with the mysid shrimp that are found to elicit some sublethal toxicity will be at risk of being labeled as endocrine toxicants without mechanistic support for this conclusion. Further, some chemicals that specifically disrupt endocrine-regulated processes in invertebrates will be missed in the Tier 1 screen if no invertebrate species is included in the screen.

1.1. Juvenoids

Juvenoids regulate various aspects of development, growth, maturation, and reproduction in arthropods (Laufer and Biggers, 2001; Quackenbush, 1986; Truman and Riddiford, 2002). Example juvenoids are juvenile hormone III in insects and methyl farnesoate

in crustaceans (Laufer et al., 1993; Riddiford, 1985). We previously established that methyl farnesoate is a sex determinant in the freshwater crustacean *Daphnia magna* (Olmstead and LeBlanc, 2002). Oocytes exposed to this hormone during ovarian oocyte maturation develop into males. We hypothesize that exposure to methyl farnesoate regulates the expression of male sex-determining genes during oocyte maturation. We also have demonstrated that some insect growth regulating (IGR) pesticides mimic the action of methyl farnesoate and alter sex ratios in daphnids (Olmstead and LeBlanc, 2003). This toxicity to daphnids is organizational, occurring only as a result of exposure of oocytes during a critical 12 h developmental window, and occurs at exposure levels several orders of magnitude below the recognized toxicity of these chemicals to vertebrates. Thus, this severe endocrine toxicity would not be detected in the currently proposed Tier 1 screen.

1.2. Ecdysteroids

Ecdysteroids are steroid hormones that, like the juvenoids, regulate various aspects of development, growth, maturation, and reproduction in arthropods (Chang et al., 2001; LeBlanc et al., 1999; Subramoniam, 2000). The ecdysteroid receptor (EcR) forms a heterodimer with the RXR homolog USP and this liganded complex binds to appropriate response elements of target genes to regulate gene activity (Yao et al., 1993, 1992). The EcR is susceptible to binding by xenobiotics, though binding typically results in the inhibition of receptor activity (antagonism) (Dinan et al., 2001a,b). Several chemicals that are known to bind to the estrogen receptor also bind to EcR (Blair et al., 2000; Dinan et al., 2001a; Sonnenschein and Soto, 1998; Soto et al., 1994; Tong et al., 1997). However, many chemicals are known to bind to the estrogen receptor that are not active towards the EcR (Arnold et al., 1996; Bulger et al., 1978; Soto et al., 1994; Tong et al., 1997). Thus, estrogen receptor-mediated activity detected in the current Tier 1 screening battery would prompt evaluation of the chronic toxicity of the chemical to mysid shrimp and toxicity determined would be inaccurately ascribed to endocrine toxicity.

The crustacean (daphnid) embryo contains a high titer of ecdysteroid early in embryo development that is likely of maternal origin (Mu and LeBlanc, 2002b). This early pool of ecdysteroid is critical to early embry-

onic development (Mu and LeBlanc, 2002a,b). Deprivation of ecdysteroid to the embryo, by inhibiting ecdysteroid synthesis in the mother or providing an ecdysteroid receptor antagonist to the embryo, results in early developmental arrest and embryo loss (Mu and LeBlanc, 2002a,b). Newly synthesized ecdysteroids by the embryo are present late in embryonic development. Deprivation or inhibition of these ecdysteroids results in late developmental abnormalities (Mu and LeBlanc, 2002c). The anti-ecdysteroidal effects of environmental chemicals can be ameliorated by coadministration of 20-hydroxyecdysone (Mu and LeBlanc, 2002a,b). This amelioration provides confirmation that the effects observed are due to endocrine toxicity.

Based upon these observations, we propose that the Tier 1 screen for endocrine-active compounds should contain a short-duration assay that will effectively detect EAT-like activity of chemicals in arthropods. Specifically, this screening assay should detect (anti)juvenoid and (anti)ecdysteroidal activity of chemicals. This screen would provide the mechanistic rationale to proceed into a Tier 2 multigenerational assay with mysid shrimp.

In the present study, we evaluated a screening assay designed for the detection of juvenoid/anti-juvenoid activity using daphnids (*Daphnia magna*). Male sex determination of offspring was used as the endpoint in this assay. With modification to include ecdysteroid-dependent endpoints, we propose that this assay will have utility in screening chemicals simultaneously for both juvenoid and ecdysteroid-dependent activities.

2. Materials and methods

2.1. Organisms

Daphnids (*Daphnia magna)* were obtained from stocks that have been maintained at North Carolina State University for over 10 years. Daphnids were cultured and used experimentally in deionized water reconstituted with 192 mg/L $CaSO_4 \cdot 2H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 μg/L selenium and 1.0 μg/L vitamin B_{12}. Cultures were maintained at a density of 40–50 brood daphnids/L culture medium. Culture medium was renewed and offspring were discarded three times weekly. Brood daphnids were discarded after 3 weeks in the culture

and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1.0 mL (~4 mg dry weight) of Tetrafin® fish food suspension (Pet International, Chesterfill, New South Wales, Australia) and 2.0 mL (1.4×10^8 cells) of a suspension of unicellular green algae, *Selenastrum capricornutum*. The algae were cultured in Bold's basal medium. Culture and experimental solutions were maintained at 20 °C under a 16 h photoperiod. These culture conditions maintained the daphnids in the parthenogenic reproductive phase with the production of all-female broods of offspring.

2.2. Chemicals

The following chemicals were evaluated with this screening assay along with the rationale for their selection.

- Juvenoids (methyl farnesoate, juvenile hormone III) are endogenous hormones in crustaceans and insects, respectively, that have been shown to stimulate male sex determination in daphnids (Olmstead and LeBlanc, 2002; Tatarazako et al., 2003).
- Pesticidal insect growth regulators (pyriproxyfen, fenoxycarb, methoprene, kinoprene) mimic the action of juvenile hormone III in insects (Dhadialla et al., 1998) and may also mimic the action of methyl farnesoate in crustaceans (Olmstead and LeBlanc, 2003; Tatarazako et al., 2003).
- All *trans*-retinoic acid functions as a hormone in vertebrates that is structurally similar to the arthropod juvenoids (Nijhout, 1998).
- Phytol is a decomposition production of chlorophyll that shares some structural similarity to the juvenoids (Kitareewan et al., 1996).
- 20-Hydroxyecdysone is an arthropod steroid hormone that was evaluated as a possible modulator of the action of juvenoids.
- Fenarimol is an agricultural fungicide that has been shown to modulate ecdysteroid levels in daphnids (Mu and LeBlanc, 2002b).
- Azadirachtin is an insect growth-regulating insecticide that interferes with hormone signaling in insects. The precise mechanism of action is not known (LeBlanc et al., 1999).
- *tert*-Amylphenol is an agricultural antimicrobial pesticide that was reported to cause developmen-

tal abnormalities in daphnids suggestive of altered sexual differentiation (Gerritsen et al., 1997).

- Dieldrin and *cis*-chlordane are cyclodiene insecticides. Dieldrin was reported to alter sex determination in daphnids (Dodson et al., 1999).
- Piperonyl butoxide is a synergist used in some pesticide formulations. Piperonyl butoxide has been reported to interfere with enzymes involved in hormone metabolism (Baldwin and LeBlanc, 1994).
- Bisphenol A and nonylphenol are ubiquitous environmental contaminants (Kolpin et al., 2002) with reported juvenoid activity (Biggers and Laufer, 2004).

The concentration of chemicals selected for use in the assay represented the greatest concentration at which the maternal daphnids exhibited no overt toxicity. Test chemicals were delivered to the exposure solutions in absolute ethanol (0.01%, v/v, final concentrations). Control solutions were provided the same concentration of ethanol. For those compounds found to be strong juvenoid agonists, the lowest concentrations assayed that provided a 100% incidence of male offspring is reported.

2.3. Screening assay: juvenoid agonist activity

Chemicals were screened for juvenoid activity based upon their ability to stimulate the production of male offspring among exposed maternal organisms. These maternal organisms were reared under conditions that promoted the production of only female offspring. We hypothesized that chemicals with juvenoid activity in insects (the insect growth regulators) or shared structural similarity with the juvenoids (retinoic acid, phytol) would elicit agonist activity. The basic experimental design for this screening assay is outlined in Fig. 1. Adult female daphnids (7–14 days old) carrying embryos in their brood chambers were selected from the cultures and placed individually in 50-ml beakers containing 40 ml media and the desired concentration of test chemical. Test solutions were changed daily and daphnids were observed daily for the release of broods of offspring. Food was provided to each beaker twice daily as 0.05 ml (~0.2 mg dry weight) of Tetrafin® fish food suspension and 0.1 mL (7×10^6 cells) of a suspension of unicellular green algae, *Selenastrum capricornutum*. Treatments were replicated 10-times (i.e., one animal per beaker, 10 beakers per treatment). Assays were terminated when all maternal daphnids in the experiment had released their third brood of offspring (typically 7–10 days).

The number of offspring present in the third brood released by each maternal daphnid was determined and sex of individual daphnids within that brood was determined. Sex of individual offspring was determined microscopically (10× magnification) with males being discerned from females by the longer first antennae
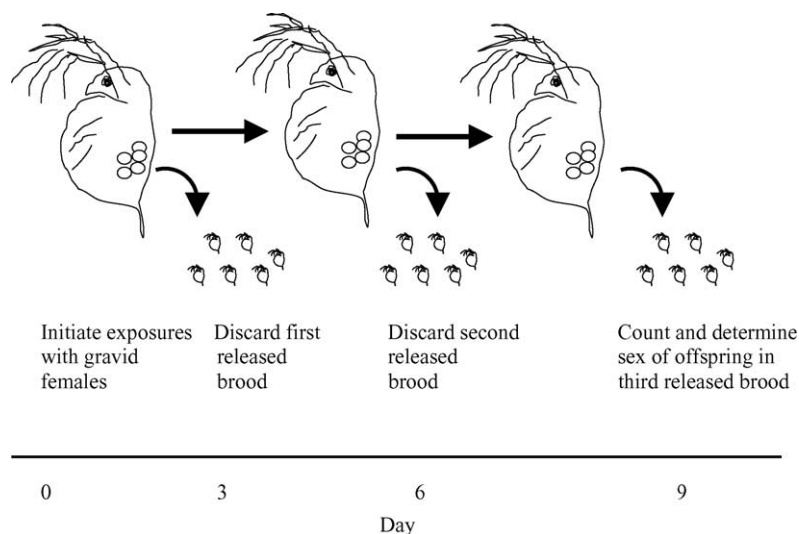

Fig. 1. Schematic representation of the screening assay experimental design used to detect juvenoid-related activity of chemicals.

(Olmstead and LeBlanc, 2000). Daphnids typically produce only female offspring under these assay conditions in the absence of exogenous juvenoid. A positive control consisting of either methyl farnesoate or pyriproxyfen was included to ensure that daphnids were appropriately responding.

Juvenoid activity was evaluated using two endpoints: percentage incidence of broods containing male offspring and percentage male offspring per male-containing brood. A brood of offspring was deemed a male-containing brood if any of the offspring within the brood were male. Percentage incidence of broods containing male offspring represented the percentage females within a treatment whose third brood contained at least one male offspring. Percentage male offspring per male-containing brood represented the proportion of offspring within the male-containing broods that was male. Both endpoints were measured in an effort to discern whether one was more robust or offered greater sensitivity. Experiments were repeated when male offspring were detected. Results are presented as the mean ± standard error of the replicate experiments. Significant differences between chemical exposures and controls were evaluated using the Wilcoxon Rank Sum Test (Gad, 1998) using SAS software (SAS, Cary, NC, USA).

2.4. Anti-juvenoid activity

Chemicals were screened for anti-juvenoid activity using the same methods as used for measuring juvenoid agonist activity except that pyriproxyfen (100 ng/L) also was added to the test solutions. Pyriproxyfen was used as the juvenoid in these experiments because similar experiments performed with methyl farnesoate resulted in potentiation as discussed below. Test chemicals were evaluated in these experiments for their ability to interfere with juvenoid (pyriproxyfen)-stimulated male offspring production. We hypothesized that weak juvenoid agonists might also function as juvenoid antagonists as observed with other hormone systems (LeBlanc, 2003; Wong et al., 1995). We also hypothesized that modulators of the ecdysone signaling pathway (20-hydroxyecdysone, fenarimol, possibly azadirachtin) might also interfere with normal juvenoid signaling since juvenoid and ecdysteroid signaling pathways sometimes interact (e.g. (Riddiford et al., 2003)). Test chemical-pyriproxyfen combinations were evaluated for significant decreases in juvenoid activity over pyriproxyfen treatment alone using the Fisher's Exact Test when analyzing the percentage male-containing broods and the Wilcoxon Rank Sum Test when analyzing the percentage males in the male-containing broods (Gad, 1998).

2.5. Juvenoid potentiator activity

Chemicals were screened for juvenoid potentiator activity using the same methods as used for measuring juvenoid agonist activity except that methyl farnesoate also was added to the test solutions at a concentration expected to marginally stimulate male offspring production (10 or 22 μg/L). Test chemicals then were evaluated for their ability to stimulate methyl farnesoate-mediated male offspring production. Methyl farnesoate was used as the juvenoid in these experiments since we hypothesized that the main mechanism of potentiation would be the inhibition of the methyl-esterase responsible for methyl farnesoate inactivation. Test chemical-methyl farnesoate combinations were evaluated for significant increases in juvenoid activity over methyl farnesoate treatment alone using the Fisher's Exact Test when analyzing the percentage male-containing broods and the Wilcoxon Rank Sum Test when analyzing the percentage males in the male-containing broods (Gad, 1998).

3. Results

3.1. Juvenoid agonist activity

Of the 18 chemicals evaluated for juvenoid agonist activity, the juvenoid hormones (methyl farnesoate and juvenile hormone III) and two of the insect growth regulators (pyriproxyfen and fenoxycarb) exhibited strong agonist activity (Table 1). The insect growth regulator methoprene and the cyclodiene insecticide dieldrin exhibited weak agonist activity with 20% of the exposed maternal daphnids producing male offspring. None of the other chemicals, at the concentrations evaluated, stimulated the production of male offspring.

Several chemicals were repeatedly evaluated to assess the incidence of false positive or negative responses. The potent agonists pyriproxyfen was assayed 13 times and all other agonists (juvenile

Table 1
Juvenoid agonist activity as measured by the ability of the test chemicals to stimulate the production of male offspring

Chemical	Concentration (μg/L)	Broods containing male offspring (%)	Male offspring per brood (%)[a]
Control	–	0	–
Methyl farnesoate	200	$100 \pm 0^{*}$	100 ± 0
Juvenile hormone III	20	$100 \pm 0^{*}$	100 ± 0
Pyriproxyfen	0.10	$100 \pm 0^{*}$	100 ± 0
Fenoxycarb	1.0	$100 \pm 0^{*}$	100 ± 0
Methoprene	310	$20 \pm 0^{*}$	42 ± 15
Kinoprene	1000	0	–
Retinoic acid	800	0	–
Phytol	10,000	0	–
Phytanic acid	300	0	–
20-Hydroxyecdysone	300	0	–
Fenarimol	1300	0	–
Azadirachtin	1.0	0	–
tert-Amylphenol	1000	0	–
Dieldrin	100	$20 \pm 0^{*}$	29 ± 18
cis-Chlordane	10	0	–
Piperonyl butoxide	300	0	–
Bisphenol A	10,000	0	–
4-Nonylphenol	200	0	–

[*] Data presented as the mean $\pm$ standard error of two experiments. Treatment was significantly different from the control ($P \leq 0.05$, Fisher's Exact Test).

[a] Data presented as the mean $\pm$ standard error (n = number of broods containing male offspring in that experiment). Data could not be subjected to statistical comparisons to controls since no male-containing broods were produced in the control treatments.

hormone III, fenoxycarb, methoprene, dieldrin) were assayed at least twice. The negative compounds kinoprene, retinoic acid, 20-hydroxyecdysone, and bisphenol A were assayed two to five times each. All assays performed with the positive responders detected juvenoid activity and all assays performed with negative responders did not detect juvenoid activity (data not shown). These results indicate that the assay is not prone to false positive or negative responses.

3.2. Anti-juvenoid activity

Five chemicals were evaluated for their ability to antagonize the juvenoid action of pyriproxyfen. These were a positive (20-hydroxyecdysone) and negative (fenarimol) modulator of the ecdysteroid signaling pathway, a putative modulator of sex differentiation (*tert*-amylphenol), and two inactive compounds (all *trans*-retinoic acid and *cis*-chlordane) that are structurally related to juvenoid-positive compounds (methyl farnseoate and dieldrin, respectively). None of the chemicals antagonized the action of pyriproxyfen (Fig. 2).

3.3. Juvenoid potentiator activity

All *trans*-retinoid acid was initially screened for its ability to potentiate the juvenoid activity associated with methyl farnesoate. This chemical shares structural similarity to methyl farnesoate but lacks the methyl–ester bond that is largely responsible for the inactivation of methyl farnesoate (Takac et al., 1997). We hypothesized that this compound might therefore bind to and inhibit the esterase responsible for inactivation of methyl farnesoate. Retinoic acid had no intrinsic juvenoid activity (see Table 1), but significantly enhanced the juvenoid activity associated with co-administered methyl farnesoate (Fig. 3).

The insect growth regulators methoprene and kinoprene also share structural similarity to methyl farnesoate but lack the methyl-ester bond and were accordingly evaluated for ability to potentiate the activity of methyl farnesoate. Methyl farnesoate, alone, did not stimulate male offspring production at the concentration used in the combination assay with methoprene. However, in combinations with methoprene, male offspring were significantly pro-
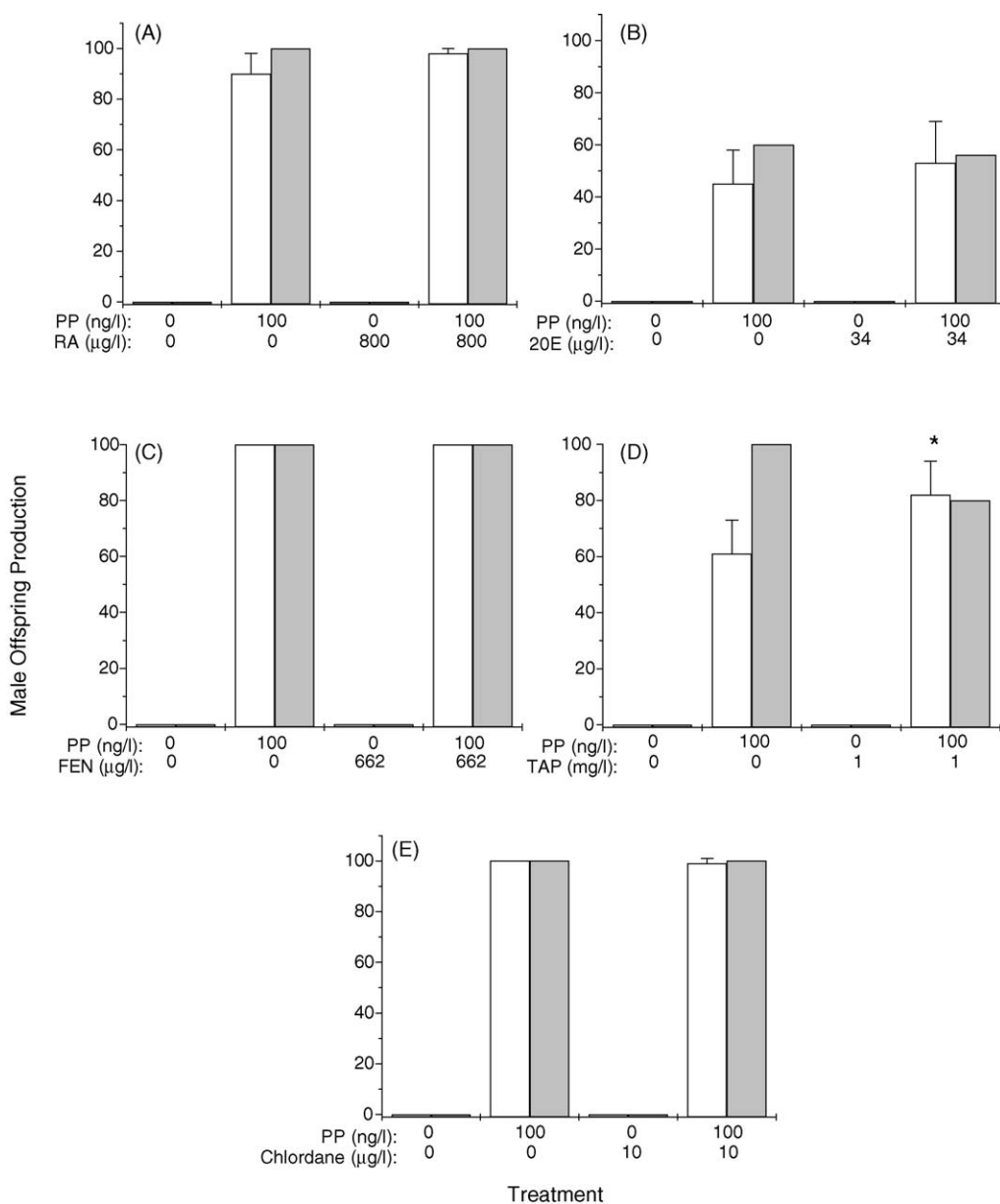
Fig. 2. Screening of five chemicals for anti-juvenoid activity. Assays were performed in the presence of 100 ng/L pyriproxyfen (PP) as the juvenoid and the ability of the test chemicals to elicit juvenoid activity (in the absence of pyriproxyfen) or antagonize the juvenoid activity associated with pyriproxifen was evaluated. Anti-juvenoid activity was evaluated with: (A) all *trans*-retinoic acid (RA); (B) 20-hydroxyecdysone (20E); (C) fenarimol (FEN); (D) *tert*-amylphenol (TAP); (E) *cis*-chlordane. White bars: percentage male offspring in male-containing broods. Error bars represent the SEM. Gray bars: percentage broods (third brood from the 10 maternal daphnids) that contained male offspring. An asterisk indicates that the test chemical significantly ($p \leq 0.05$) altered the juvenoid activity as compared to that measured with pyriproxyfen alone.
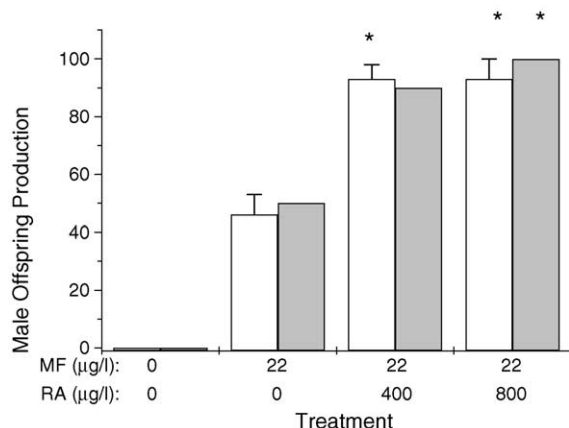
Fig. 3. Potentiation of juvenoid activity associated with methyl far-
nesoate (MF) by all *trans*-retinoic acid. White bars: percentage male
offspring in male-containing broods. Error bars represent the S.E.M.
Gray bars: percentage broods (third brood from the 10 maternal daph-
nids) that contained male offspring. An asterisk indicates that the test
chemical significantly ($p \leq 0.05$) elevated juvenoid activity over that
measured with methyl farnesoate alone. As asterisk denotes a signif-
icant ($p \leq 0.05$) difference from methyl farnesoate treatment alone.

duced (Fig. 4A). Kinoprene, which was not found
to elicit juvenoid activity in the agonist assay, also
significantly elevated juvenoid activity associated
with methyl farnesoate (Fig. 4B). These observations
are consistent with our assertion that some structural
analogs to methyl farnesoate can potentiate the action
of methyl farnesoate though they do not establish
the mechanism responsible for the interaction. The
phenolic environmental contaminant bisphenol A also
potentiated the action of methyl farnesoate (Fig. 4C),
although alone, it had no detectable juvenoid activity.

4. Discussion

The general hypothesis was tested in this study
that a screening assay could be employed to detect
juvenoid-related endocrine-modulating activity in an
invertebrate. Such an assay was developed and eval-
uated that detected juvenoid agonist and potentiator
activity. Anti-juvenoid activity was not detected among
the chemicals evaluated. This lack of detection may
reflect a weakness in the experimental design. For
example, antagonistic activity was evaluated in this
assay against a potent juvenoid, pyriproxyfen, that was
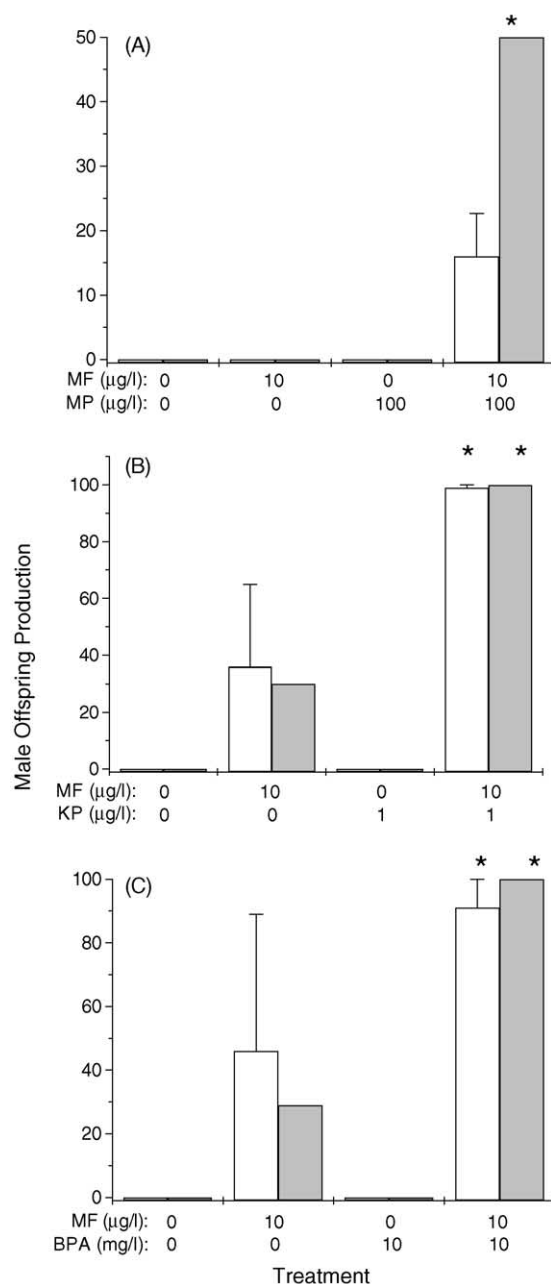present in the assay at a level that typically caused a

Fig. 4. Potentiation of juvenoid activity associated with methyl far-
nesoate (MF) by: (A) methoprene (MP); (B) kinoprene (KP); and,
(C) bisphenol A (BPA). White bars: percentage male offspring in
male-containing broods. Error bars represent the S.E.M. Gray bars:
percentage broods (third brood from the 10 maternal daphnids) that
contained male offspring. An asterisk indicates that the test chemical
significantly ($p \leq 0.05$) altered the juvenoid activity as compared to
that measured with methyl farnesoate alone.

100% incidence of male offspring. Perhaps antagonists would be more readily detected at a lower concentration of pyriproxyfen that stimulated only a partial incidence of male offspring. The difficulty in performing the assay at such a concentration of pyriproxyfen stems from the steep concentration-response relationship for this potent juvenoid agonist (Olmstead and LeBlanc, 2003). A subtle difference in the concentration of pyriproxyfen used in the assay can result in a change from 100% incidence of male offspring to a 0% incidence. Nonetheless, results from the study support the hypothesis that chemicals can be effectively screened for juvenoid-modulating activity.

Among the chemicals evaluated, agonist activity was detected with juvenile hormone III along with its analogs pyriproxyfen, fenoxycarb, and methoprene. These observations corroborate previous reports of the ability of these compounds to stimulate male offspring production in daphnids (Olmstead and LeBlanc, 2003; Tatarazako et al., 2003) and provides further support to the hypothesis that a crustacean juvenoid hormone, such as methyl farnesoate or an analog thereof, is responsible for regulating sex determination in these organisms. However, not all juvenoids evaluated functioned as agonists in this assay. The insect growth regulator kinoprene did not stimulate male offspring production under these conditions.

All *trans*-retinoic acid and phytol, which share terpenoid-like structural characteristics with methyl farnesoate, were not found to elicit juvenoid activity in the screening assay. These observations further demonstrate the specificity of the assay and provide additional evidence to suggest that the putative methyl farnesoate receptor has rather stringent agonist-binding requirements.

Methyl farnesoate is inactivated by the action of methyl-esterases in peripheral tissues of crustaceans (Homola and Chang, 1997; Takac et al., 1997). Chemicals that inhibit this esterase activity could serve to elevate levels of active endogenous or administered methyl farnesoate by blocking its inactivation. The juvenoid analogs methoprene and kinoprene, though weakly active or deficient in their ability to mimic the action of methyl farnesoate, were effective in potentiating the action of methyl farnesoate. These compounds share structural similarity to methyl farnesoate and may compete for binding to the inactivating methyl-esterase enzyme thus blocking the metabolism

of methyl farnesoate and increasing its accumulation within the organisms. Interestingly, the xenobiotic bisphenol A which does not share significant structural similarity to methyl farnesoate and exhibited no discernible intrinsic juvenoid activity also was an effective juvenoid potentiator. If inhibition of the methyl farnesoate methyl-esterase is the mechanism of potentiation, results suggest that this enzyme exhibits rather promiscuous binding activity and may serve as a major target for juvenoid modulating activity of environmental chemicals. Chemicals with inhibitory activity towards juvenile hormone esterases of insects have been sought for potential use as insecticidal agents (Szekacs et al., 1990). Results from the present study suggests that such agents may lack the level of insect specificity ascribed to them (Wheelock et al., 2003), and may also interfere with endocrine signaling processes in crustaceans.

Bisphenol A was previously reported to exhibit juvenoid activity using the metamorphosis of polychaete (*Capitella*) larvae as the assay endpoint (Biggers and Laufer, 2004). Bisphenol A induced metamorphosis with an EC50 of 11 µg/L while in the daphnid assay, bisphenol A did not exhibit juvenoid activity at exposure concentrations as high as 10,000 µg/L (Table 1, and (Mu et al., 2005)). The reason for this difference in response is not obvious, though it may indicate that different juvenoid hormones are operative in polychaetes and crustaceans resulting in sensitivity differences between species. Alternatively, polychaetes, but not daphnids, might biotransform bisphenol A to a juvenoid-active metabolite. Finally, polychaete larvae may have significant levels of endogenous juvenoid as compared to daphnids and the juvenoid activity detected with bisphenol A may have been due to potentiation of this endogenous juvenoid as we did observe with the daphnid assay.

The production of male offspring in these experiments was assessed as both the percentage incidence of broods containing male offspring and the percentage male offspring per male-containing brood. Both endpoints provided comparable measures of juvenoid activity and neither endpoint provided evidence of superiority. Since both endpoints can be evaluated with the same effort, we recommend that both be measured and presented when using this assay.

Dodson et al., (1999) reported that exposure of maternal daphnids to dieldrin concentrations ≥100 µg/L decreased the proportion of male offspring

produced. These observations were made under conditions in which the daphnids were naturally producing a low incidence of male offspring suggesting that endogenous methyl farnesoate levels were somewhat elevated in these organisms. In our screening assay, dieldrin (100 μg/L) exhibited weak juvenoid activity when administered with no exogenous methyl farnesoate. Taken together, these observations suggest that dieldrin functions as a mixed agonist/antagonist (LeBlanc, 2003). In the absence of methyl farnesoate (agonist assay in present study), dieldrin may bind and weakly activate the methyl farnesoate receptor. In the presence of low methyl farnesoate levels ((Dodson et al., 1999) study in which daphnids were naturally producing a low incidence of males), dieldrin may have successfully competed with methyl farnesoate for partial occupancy of the methyl farnesoate receptor resulting in a net negative effect on receptor activation (i.e., displacement of a strong activator by a weak activator). Hormone receptor ligands have been shown to act as either agonists or antagonists depending upon the concentration of competing natural ligand (Wong et al., 1995).

Juvenoid and ecdysteroid signaling pathways are closely coordinated in arthropods. As developmental hormones, juvenoids modulate ecdysteroid signaling (Nijhout, 1998). In this screening assay an ecdysteroid (20-hydroxyecdysone), an ecdysteroid synthesis inhibitor (fenarimol), and an anti-ecdysteroid having an occult mechanism of action (azadirachtin) were evaluated for possible interaction with the juvenoid signaling pathway. None of these compounds were detected in the screening assay as having juvenoid-modulating activity. These results are consistent with previous observations that while juvenoids modulate ecdysteroid activity, ecdysteroids do not modulate juvenoid activity (Mu and LeBlanc, 2004).

Among the 18 compounds evaluated in this screening assay, six registered positive as juvenoid agonists and all six of the compounds evaluated as juvenoid potentiators registered positive. Among the potentiators were four compounds (all *trans*-retinoic acid, kinoprene, 4-nonylphenol, and bisphenol A) that were not detected based upon agonist activity. Among the six juvenoid agonists detected, only dieldrin likely would have registered positive in the EDSTAC-recommended Tier 1 screening battery as we are not aware of any of the other compounds ever having been shown to elicit

estrogen, androgen, or thyroid hormone-like activities in vertebrates. Among the detected potentiators, 4-nonylphenol and bisphenol A would likely be detected in the proposed screen due to their estrogenic activity (Vivacqua et al., 2003). However, demonstrated estrogenic activity in vertebrates would not provide the mechanistic rationale for evaluating these chemicals in an invertebrate under Tier 2 testing. Therefore, without a suitable Tier 1 screen to discern juvenoid potentiator activity, effects observed with mysids under Tier 2 testing would remain of unknown etiology.

Finally, with modification, this proposed screening holds promise for evaluating ecdysteroid-related activities of chemicals as well as juvenoid-related activities. For example, we are exploring the feasibility of evaluating daphnids – either maternal organisms or their offspring – at the end of the assay for induction of ecdysteroid responsive genes such as HR3, HR38, and E75A/B (El Haj et al., 1997; Hiruma and Riddiford, 2004; White et al., 1997). Such induction could serve to detect ecdysteroid-agonist activity associated with chemicals. Such analyses are feasible in a screening format by adopting real time RT-PCR techniques in conjunction with high-throughput assay methods (Grunst, 2001; Overbergh et al., 1999). The incorporation of developmental endpoints that are indicative of anti-ecdysteroidal activity of chemicals (Mu and LeBlanc, 2002a,b) are also under evaluation. The successful integration of these multiple endpoints into a single screening assay would provide much needed mechanistic support for endocrine activity of chemicals towards arthropods. Such information would then provide a rationale for progression into a Tier 2 test with mysid shrimp.

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