SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 24, No. 1, pp. 146–152, 2005
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Printed in the USA
0730-7268/05 $12.00 + .00

# COVERT SIGNAL DISRUPTION: ANTI-ECDYSTEROIDAL ACTIVITY OF BISPHENOL A INVOLVES CROSS TALK BETWEEN SIGNALING PATHWAYS

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(*Received* 6 *February* 2004; *Accepted* 17 *June* 2004)

**Abstract**—Bisphenol A is a key industrial chemical used in the manufacture of polycarbonate plastics and other products. Several recent reports ascribe toxicological properties to this compound that have been attributed to the disruption of endocrine-related processes. In the present study, the toxicity of bisphenol A was definitively characterized in the water flea (*Daphnia magna*) in an effort to discern whether this compound may elicit endocrine toxicity in an invertebrate species and to establish the mechanism by which this toxicity is elicited. The ability of bisphenol A to interfere with two ecdysteroid-dependent physiological processes—molting and embryonic development—was evaluated. Bisphenol A elicited antiecdysteroidal activity as indicated by its prolongation of the intermolt period and interference with embryonic development. This apparent antiecdysteroidal activity was not due to reduced availability of endogenous ecdysteroid nor due to ecdysteroid-receptor antagonism. The ability of bisphenol A to elicit antiecdysteroidal activity by functioning as a juvenoid hormone was next evaluated. Bisphenol A, alone, did not elicit juvenoid activity. However, bisphenol A did enhance the activity of the crustacean juvenoid hormone methyl farnesoate. A definitive assessment of the effects of bisphenol A on the reproductive capacity of daphnids revealed a concentration–response relationship that extended at least one order of magnitude below exposure levels that were overtly toxic to the maternal organisms. These results demonstrate that bisphenol A is chronically toxic to daphnids, probably through its ability to interfere with ecdysteroid/juvenoid regulated processes. However, effects are elicited at levels that are not likely to pose environmental concern.

**Keywords**—Crustacean Pollutant Reproduction Steroid Terpenoid

## INTRODUCTION

The characterization of endocrine toxicity has focused primarily on the effects of environmental chemicals on androgen, estrogen, and thyroid hormone dependent-processes. This emphasis is predicated by the fact that these three hormone systems are intricately involved in regulating development, growth, and reproduction in vertebrates and thus provide a good first attempt at detecting and mitigating endocrine-disrupting toxicity (U.S. Environmental Protection Agency Endocrine Disruptor Screening Program Overview; http://www.epa.gov/scipoly/oscpendo/edspoverview/index.htm). However, the use of these signaling molecules in invertebrates is equivocal. Conversely, other signaling molecules are clearly operative in invertebrates but are not physiologically relevant in vertebrates [1].

Among these invertebrate hormones are ecdysteroids. Ecdysteroids represent a class of steroid hormones in arthropods (insects, crustaceans, and some minor phyla) that regulate many aspects of development, growth, and reproduction that are associated with androgens, estrogens, and thyroid hormone in vertebrates. For example, embryonic development in crustaceans is regulated in part by ecdysteroids [2]. Growth in arthropods requires periodic loss of the exoskeleton and replacement with a larger cuticle. This molting process is regulated by ecdysteroids [3], and thus, these hormones are permissive of growth. Ecdysteroids also have been shown to regulate aspects of vitellogenesis and spermatogenesis and therefore also function as reproductive hormones [4–6].

Ecdysteroids function through receptor activation in a manner orthologous to the vertebrate thyroid hormone receptor. Functional activation of the ecdysteroid receptor (EcR) is mediated in part through its dimerization with a partner protein ultraspiracle and binding of the ecdysteroid ligand (typically 20-hydroxyecdysone) [7,8]. Ultraspiracle protein is homologous to the vertebrate retinoid-X receptor [8,9]. This ligand–dimer complex then regulates activity of ecdysteroid-responsive genes by interacting with *cis*-acting elements of the genes.

Environmental chemicals that have the potential to bind EcR as agonists or antagonists may elicit toxicity unique to arthropods. Indeed, some insecticides, such as tebufenozide, elicit highly specific effects on target insects based on their ability to bind the EcR as agonists [10]. We recently reported that testosterone functions as an EcR antagonist [11] and accordingly interferes with the ecdysteroid-regulated processes of molting and embryonic development in the crustacean *Daphnia magna* [12]. The industrial chemical and environmental contaminant bisphenol A was reported to function as a weak ecdysteroid antagonist in an insect cell-based assay [13]. However, we are aware of no reports of bisphenol A eliciting toxicity to an arthropod species by functioning as an antiecdysteroid.

Chemicals that lower endogenous ecdysteroid levels or function as ecdysteroid antagonists in daphnids cause abnormal embryonic development, resulting in reduced production of viable offspring [2,11]. The mechanistic specificity of such toxicity may render arthropods particularly sensitive to the toxicity of such materials. We hypothesized that bisphenol A would interfere with ecdysteroid-dependent embryonic development of daphnids, resulting in reduced fecundity.

Bisphenol A is an industrial chemical used in the manufacture of polycarbonate plastics, epoxy resins, and other products. This compound has been shown to interact with the es-

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trogen receptor as an agonist and elicit estrogen-like activity in vivo [14–17]. This compound also has been shown to interact with the mammalian androgen receptor in an antagonistic manner [18]. The material is ubiquitous in the aquatic environment, with concentrations typically measured in the parts per billion range (discussed in [19]).

## MATERIALS AND METHODS

### *Daphnids*

Daphnids were cultured and experimentally maintained in deionized water reconstituted with 192 mg/L $CaSO_4 \cdot H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 mg/L selenium, and 1.0 mg/L vitamin $B_{12}$. Cultures were maintained at a density of 45 brood daphnids/L culture medium. Culture medium was renewed and offspring were discarded three times weekly. Brood daphnids were discarded after three weeks in the culture and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1.0 ml (~4 mg dry wt) of Tetrafin® fish food suspension (Pet International, Chesterfill, NSW, Australia) and 2.0 ml ($1.4 \times 10^8$ 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.

### *Ecdysteroid-dependent endpoints measured during continuous exposure*

Experiments were performed to determine whether bisphenol A elicited antiecdysteroidal activity in vivo as indicated by alterations in the ecdysteroid-dependent endpoints: intermolt duration and embryonic development.

*Intermolt duration.* Individual female daphnids (<1 h old) were exposed to each of 20 concentrations of bisphenol A (Aldrich Chemical, Milwaukee, WI, USA) with each concentration being 90% of the next highest concentration. Exposure concentrations were selected in which the highest concentration approximated the no-discernible-effect concentration as measured during preliminary experiments. Exposures were conducted in 50-ml beakers containing 40 ml of media. All treatments, including controls, contained the same amount (0.01%) of vehicle (absolute ethanol, AAPER, Shelbyville, KY, USA) used to dissolve the bisphenol A. Controls for each experiment consisted of 10 beakers each containing a single daphnid. Daphnids were provided food (0.05 ml [~0.2 mg dry wt] of Tetrafin fish food suspension and 0.1 ml [$7 \times 10^6$ cells] of a suspension of unicellular green algae, *S. capricornutum*) and test solutions were renewed every 24 h. Vessels were examined hourly, during the 10-h period during which molting occurred, for the presence of a molted exoskeleton, and time of molting was recorded to the nearest hour for each daphnid. The duration of the first intermolt period (time between birth and molting to the nearest hour) for each daphnid was plotted against exposure concentration and results judged to best conform to either a single-segmented line or a two-segmented line. Three possible relationships were considered. First, a single-segmented line having no slope was considered. This would be indicative of the chemical having no effect on intermolt duration. Second, a single-segmented line having a significant slope and a significant positive relationship between exposure concentration and intermolt duration was considered. This would indicate an anti–ecdysteroid-like effect with no threshold concentration. Third, a two-segmented line consisting of the first relationship followed by the second relationship was considered. This would be indicative of anti-ecdysteroidal effects with a threshold concentration.

The ability of 20-hydroxyecdysone (Sigma Chemical, St. Louis, MO, USA) to protect daphnids against the effects of bisphenol A on molting was assessed using the same basic procedures as described above except that the effect of each treatment was evaluated using the mean ± standard deviation response of eight neonatal daphnids. Both bisphenol A and 20-hydroxyecdysone were delivered to the media in ethanol at a final total ethanol concentration of 0.01%. Control solutions also contained this amount of ethanol. Significant differences among treatments were evaluated using analysis of variance and Dunnett's *t* test [20] at $p \leq 0.05$.

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

### *Isolated embryo exposures*

Embryos of the same age (as indicated in the specific experiments), were removed from untreated maternal daphnids as described previously [12]. Embryos were individually and randomly assigned to wells of 96-well microtiter plates along with 200 μl of medium containing various concentrations of bisphenol A. All embryos, including controls, were exposed to the same concentration of ethanol vehicle (0.01%). Embryos were incubated at 20°C under a 16-h photoperiod and were examined microscopically every 24 h. Embryos were scored for stage of development and any abnormalities were noted. The percentage of embryos that exhibited developmental abnormalities was determined when development of the control daphnids was complete. The number of embryos used per treatment level are indicated in the *Results* section for individual experiments.

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

### *Male sex determination*

Male sex determination is a juvenoid-regulated process in daphnids [21]. Juvenoid activity of bisphenol A was measured by its ability to stimulate the production of male offspring by treated maternal organisms. Maternal daphnids were exposed to concentrations of bisphenol A alone or in the presence of the juvenoid hormone methyl farnesoate (Echelon Biosciences, Salt Lake City, UT, USA) during ovarian oocyte maturation, and sex of the resulting progeny was determined. Adult female daphnids carrying embryos in their brood chambers were selected from the cultures and placed individually in 50-ml beakers containing 40 ml media. Forty-eight hours after the release

of the brood of offspring, daphnids were transferred to media containing the appropriate concentrations of the test chemicals. The daphnids were maintained in this solution for 24 h, which encompassed the sex-determination period of ovarian oocyte maturation. Daphnids then were transferred to chemical-free medium and maintained until the brood of offspring exposed to test chemical(s) in the ovary was released. Food was provided to each beaker twice daily as described above. Daphnids typically produce only female offspring under these culture conditions in the absence of exogenous juvenoids, but will produce males if exposed to juvenoids. Sex of individual offspring was determined microscopically, with males being discerned from females by the longer first antennae [22]. Significant differences among treatments were assessed by analysis of variance and the appropriate comparison test.

### *Maternal fecundity*

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

### *Acute toxicity assessments*

The acute toxicity of bisphenol A to daphnids was evaluated using standard procedures [23]. Daphnids (<24 h old) were exposed to concentrations of the chemical for 48 h and adverse effects (immobilization, lethargy, death) were determined. Exposure concentrations were selected that were expected to bracket the median effective concentration (EC50) value, based on preliminary experiments. For each treatment level, two 50-ml beakers containing 40 ml of solution and 10 daphnids were used. The chemicals were delivered to the media (same as culture media) at the desired concentration dissolved in absolute ethanol. All solutions and controls for a specific experiment contained the same concentration of ethanol (≤0.01%). Vessels were maintained in a temperature- and light-controlled incubator as described for cultures. After 48 h, daphnids were examined for effects and EC50 values and accompanying 95% confidence intervals were calculated using Origin software (MicroCal Software, Northhampton, MA, USA).

## RESULTS

### *Antiecdysteroidal activity of bisphenol A*

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

### *Protection by 20-hydroxyecdysone*

Among the mechanisms by which a chemical may elicit anti-ecdysteroidal activity are the lowering of endogenous ecdysteroid titers and competitive antagonism at the ecdysone receptor. In either case, the anti-ecdysteroidal effect of the chemical can be ameliorated by the provision of exogenous 20-hydroxyecdysone [2,11]. Experiments were therefore performed to determine whether daphnids could be rescued from the adverse effects of bisphenol A by coexposure to 20-hydroxyecdysone. Exposure of daphnids to 9.0 mg/L bisphenol A through the first intermolt period significantly increased the duration of this period (Fig. 3). However, coexposure to concentrations of 20-hydroxyecdysone had no protective effect on this action of bisphenol A (Fig. 3). Similarly, exposure of isolated embryos to bisphenol A increased the incidence of developmental abnormalities, but coexposure to 20-hydroxyecdysone had no protective effect toward the embryos (Fig.

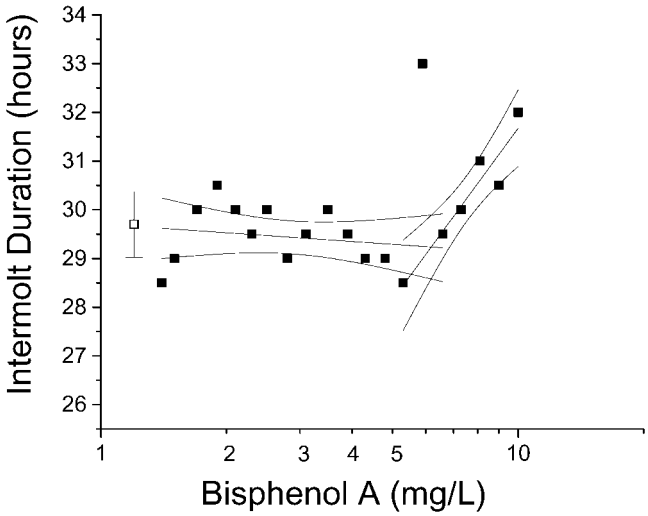


Fig. 1. Intermolt duration between birth and the first molt of neonatal daphnids exposed to concentrations of bisphenol A. Each data point represents an individual daphnid. The data were fit to a two-segmented line with associated 95% confidence intervals. Mean (± standard deviation) performance of 10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

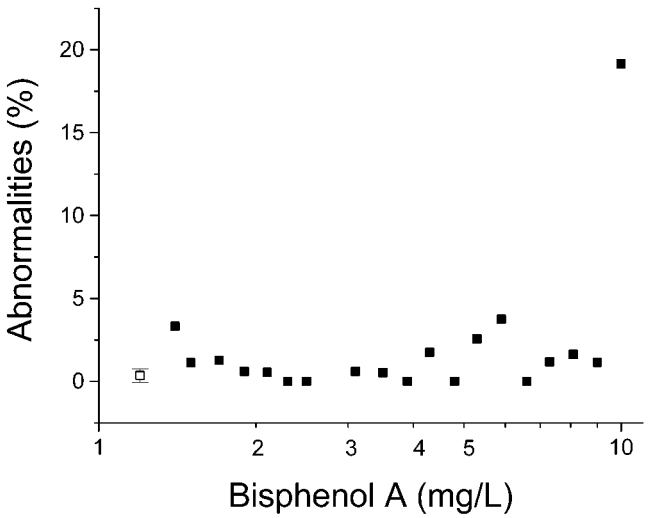


Fig. 2. Percentage neonates with developmental abnormalities during exposure to concentrations of bisphenol A (94–205 neonates per female were evaluated). Each data point represents the percentage abnormal neonates produced by a single maternal daphnid exposed to the indicated concentration of bisphenol A. Mean (± standard deviation) performance of 10 unexposed (control) daphnids is depicted by the open square.

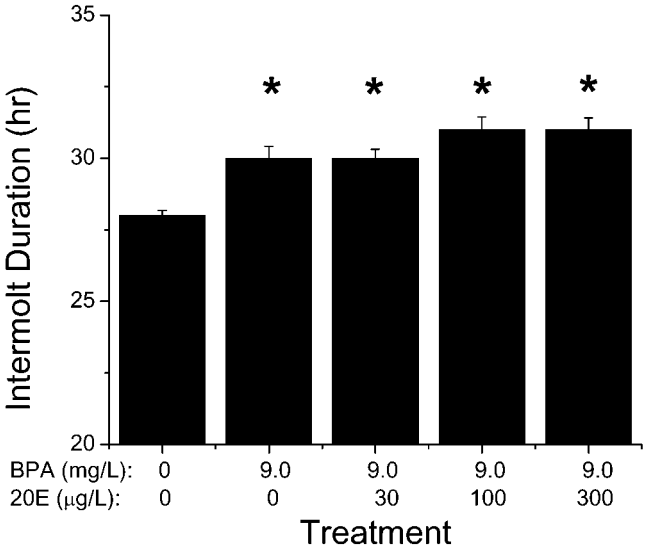


Fig. 3. Influence of 20-hydroxyecdysone (20E) exposure on delayed molting caused by bisphenol A (BPA). Neonatal daphnids (<1 h old) were individually exposed to 9.0 mg/L bisphenol A alone or in combination with concentrations of 20E. The duration of the first intermolt period was measured as described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

4). Rather, 20-hydroxyecdysone enhanced the developmental toxicity of bisphenol A (Fig. 4). During the performance of these experiments, it also became evident that susceptibility of embryos to the developmental toxicity of bisphenol A was inversely related to age of the embryos (Fig. 4A versus B, Table 1).

### *Juvenoid activity of bisphenol A*

Juvenoid hormones are integral signaling molecules in arthropods, including crustaceans, that can negatively regulate ecdysteroid signaling [24]. Thus, juvenoids can be viewed as having anti-ecdysteroid activity. Experiments were performed to determine whether the apparent anti-ecdysteroidal activity of bisphenol A could be due to intrinsic juvenoid activity of this compound. The ability of bisphenol A to stimulate the production of male offspring, a highly specific juvenoid-regulated process in daphnids, was evaluated. Bisphenol A was unable to stimulate the production of male offspring when maternal daphnids were exposed to this compound alone (Fig. 5). However, exposure to bisphenol A along with the juvenoid methyl farnesoate stimulated the production of male offspring to a level greater than that caused by the methyl farnesoate alone (Fig. 5). This effect suggests that the anti-ecdysteroidal activity of bisphenol A could be due to its ability to synergize with endogenous juvenoids.

### *Effect of bisphenol A on general fecundity*

Results from these experiments suggested that bisphenol A could reduce overall fecundity of daphnids by interfering with embryonic development, resulting in a decrease in the production of viable offspring. This potential was evaluated by continuously exposing maternal daphnids to concentrations of bisphenol A and evaluating the production of viable offspring by these organisms. The number of offspring produced by bisphenol A-exposed daphnids progressively declined with increasing bisphenol A concentration over the range of concentrations that overtly elicited anti-ecdysteroidal activity (2.5–10 mg/L) (Fig. 6). Results suggest that the threshold concentration (i.e., intercept with the dashed control line in Fig. 6) exists at approximately 1.3 mg/L bisphenol A.

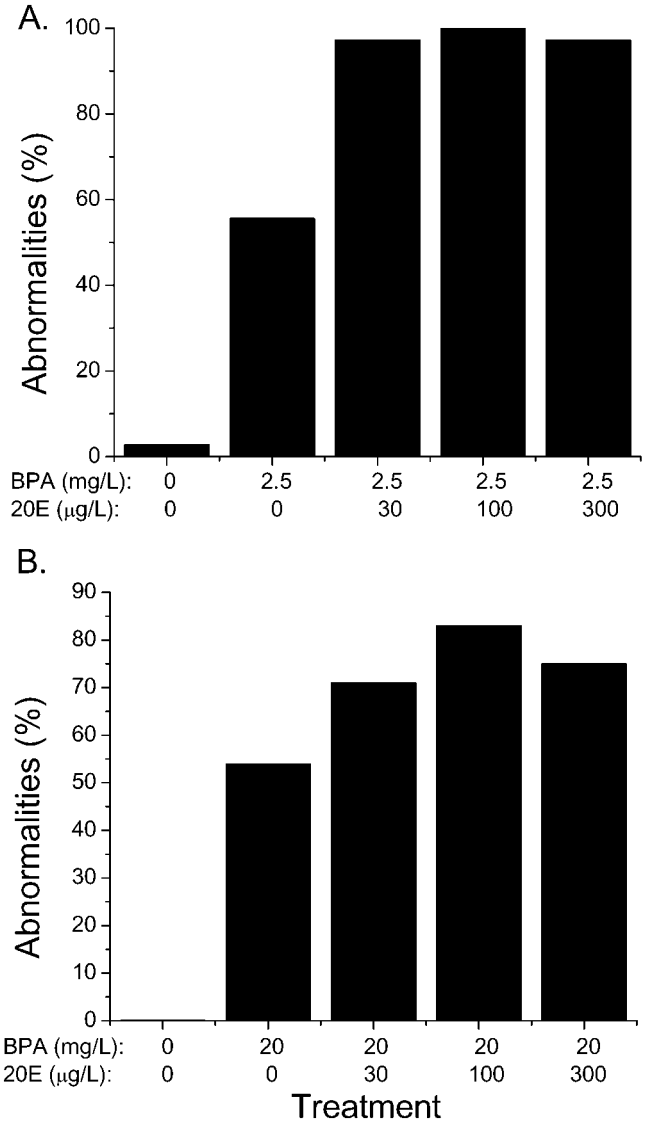


Fig. 4. Influence of 20-hydroxyecdysone (20E) exposure on developmental abnormalities caused by bisphenol A (BPA). Embryos were removed from maternal daphnids 6.5 h (**A**) or 18 h (**B**) after deposition from the ovaries to the brood chamber and individually exposed to either 2.5 mg/L (**A**) or 20 mg/L (**B**) bisphenol A in the presence or absence of 20-hydroxyecdysone. The incidence of developmental abnormalities was assessed upon completion of development among control embryos (~80 h). Each treatment consisted of 23 to 24 individual embryos.

### *Acute:chronic ratio of bisphenol A*

The ratio of the acute toxicity of a chemical expressed as its EC50 value and its threshold concentration, as measured during full life-cycle exposure, is often used as a measure of the propensity of a chemical to elicit chronic toxicity [25]. The EC50 value for bisphenol A with daphnids was 16 mg/L (95% confidence interval, 15.9–16.4) providing an acute: chronic ratio (16/1.3) of 12. Consistent with this specific mode of reproductive toxicity (anti-ecdysteroidal activity), an acute: chronic ratio of >10 is typically indicative of chronic toxicity

Table 1. Susceptibility of variously aged embryos to the developmental toxicity of bisphenol A. Age denotes the length of time that embryos were allowed to develop in the brood chambers of maternal organisms following transfer from the ovaries. Embryos were removed at the designated time and allowed to continue development ex vivo while exposed to the indicated concentration of bisphenol A. The incidence of developmental abnormalities was assessed upon completion of development among the respective control embryos (~72 h). Each treatment consisted of 19 to 36 embryos

| Experiment | Embryo age (h) | Bisphenol A (mg/L) | Developmental abnormalities (%) |
|---|---|---|---|
| 1 | 6.5 | 0 | 0 |
| | | 2.5 | 56 |
| 2 | 8.0 | 0 | 0 |
| | | 10.0 | 35 |
| 3 | 8.0 | 0 | 0 |
| | | 5.0 | 17 |
| | | 10.0 | 21 |
| 4 | 12 | 0 | 0 |
| | | 5.0 | 0 |
| | | 10.0 | 0 |
| 5 | 18 | 0 | 0 |
| | | 20.0 | 54 |

caused by a mechanism of toxicity distinct from that responsible for acute toxicity [26].

## DISCUSSION

Results from the present study demonstrate that bisphenol A elicits anti-ecdysteroidal activity in the crustacean *D. magna*. This effect appears to be a consequence of the ability of bisphenol A to synergize with endogenous juvenoid hormone

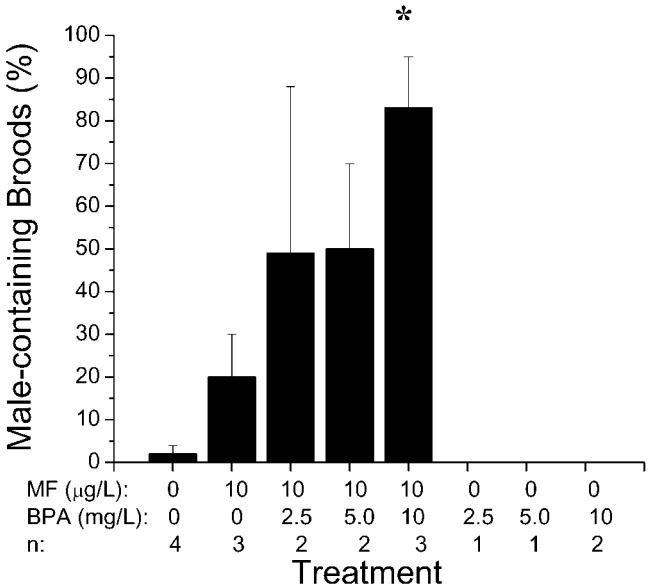


Fig. 5. Incidence of male-sex determination among offspring produced by maternal daphnids exposed to methyl farnesoate (MF), bisphenol A (BPA), or combinations thereof. Maternal daphnids (7–10 per treatment) were exposed to the chemicals, as indicated, through a complete period of oocyte/embryo maturation. Resulting neonates were sexed upon release from the maternal daphnids and the percentage broods that contained male offspring were determined for each treatment. The n denotes the number of times the experiment was repeated. Data are presented as the mean and standard error of the replicate experiments. An asterisk denotes a significant difference ($p \leq 0.05$, analysis of variance following arcsin transformation) from untreated daphnids and daphnids treated with MF alone (Student's *t* test).

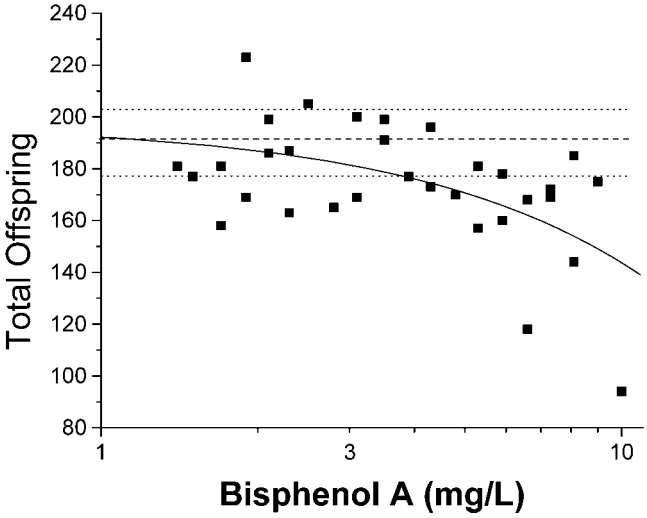


Fig. 6. Total offspring produced among daphnids continuously exposed to concentrations of bisphenol A for 21 d. Each data point represents the total offspring produced by a single maternal daphnid. Dashed lines represent the mean and standard deviation number of offspring produced by 10 control daphnids. Data were fit to a second-order regression line.

(methyl farnesoate), resulting in juvenoid-mediated inhibition of ecdysteroid signaling. This effect may contribute to reproductive toxicity associated with the compound. Bisphenol A was shown to elicit anti-ecdysteroidal activity in cultured insect cells, by interfering with ecdysteroid-dependent arrest of cell proliferation, with an EC50 of 23 mg/L [13]. In vivo results from the present study were remarkably consistent with this in vitro assessment, where overt anti-ecdysteroidal effects on daphnid molting and embryonic development were evident in the 5 to 10 mg/L range.

Toxicity of bisphenol A to arthropods has previously been reported that is consistent with the anti-ecdysteroidal/juvenoid activity reported in the present study. Molting was delayed in the blood worm (*Chironomus riparius*) at an exposure level of 1 mg/L bisphenol A [27]. Bisphenol A was reported to inhibit naupliar development of the copepod *Acartia tonsa*, with an EC10 of 0.22 mg/L [28], and mouthpart development in *C. riparius* at 1 mg/L [27].

The inability of exogenous 20-hydroxyecdysone to protect against the anti-ecdysteroidal toxicity of bisphenol A argues that the precise mechanism of toxicity does not involve depletion of endogenous ecdysteroid levels or competitive antagonism at the ecdysteroid receptor. We have shown that toxicity involving both of these mechanisms can be ameliorated by coadministration of 20-hydroxyecdysone [2,11]. This observation stands in contrast with results from cell-free receptor binding assays, where bisphenol A was shown to compete with the ecdysteroid ponasterone A for binding to the ecdysone receptor [13]. This discrepancy may indicate that bisphenol A elicits multiple mechanisms of anti-ecdysteroidal activity and that receptor antagonism is not the predominant mechanism in vivo.

Bisphenol A was shown to enhance activity of the crustacean juvenoid hormone methyl farnesoate. Similarly, we have previously reported synergy when daphnids were treated with methyl farnesoate along with the synthetic juvenile hormone analogs pyriproxyfen or methoprene [29]. Methyl farnesoate is inactivated through hydrolysis of an ester bond on the molecule [30]. We suspect that bisphenol A, pyriproxyfen,

and methoprene also bind to the methyl farnesoate esterase; however, these compounds may lack the ester bond that is hydrolyzed by the enzyme. These more stable compounds could competitively inhibit the hydrolysis of methyl farnesoate, causing levels of the hormone to increase in the organisms. Embryos may be particularly dependent on methyl farnesoate hydrolysis during ecdysteroid-sensitive developmental periods (see [31]). Inhibition or suppression of the embryonic esterase during these periods by bisphenol A would result in the developmental abnormalities observed. The increased susceptibility of embryos to bisphenol A (Table 1) is consistent with this postulate, as early embryonic development is highly dependent on ecdysteroids [2]. Additional research is warranted to establish whether bisphenol A enhances juvenoid signaling in embryos by inhibiting juvenoid esterase activity.

Anti–ecdysteroid-like activity of juvenoids has been previously demonstrated in arthropods. For example, the juvenoid methoprene has been shown to block ecdysteroid-mediated induction of heat-shock proteins [32] and micro-RNAs [33]. RNA synthesis can be modulated by 20-hydroxyecdysone in the silk gland of *Galleria mellonella*. Cotreatment with juvenile hormone can block this action of ecdysteroids on RNA synthesis [34]. Juvenile hormones have been shown to suppress ecdysteroid-regulated molting of the lobster (*Homarus americanus*) larvae [35] and naupliar development of the copepod *A. tonsa* [28]. We recently have shown that insecticidal juvenile hormone analogs function as anti-ecdysteroids in a manner comparable with bisphenol A described in the present study [24]. These compounds interfered with embryo development with the same developmental abnormalities as described for bisphenol A. This latter observation lends additional support to the conclusion that the mechanism of anti-ecdysteroidal activity of bisphenol A is due to its ability to enhance endogenous juvenoid activity.

Results of the present study provide a mechanism by which bisphenol A can elicit reproductive toxicity to crustaceans. Results further demonstrate that reproductive toxicity of this compound can be detected at exposure concentrations approximating one order of magnitude below levels that elicit acute toxicity. While these results are indicative of chronic toxicity of this compound, exposure concentrations at which these effects are elicited remain significantly above expected environmental levels. In a survey of 139 streams across the United States that were downstream of significant urbanization or agriculture, bisphenol A was detected in 41% of the sites. The median concentration detected was 0.14 μg/L and the maximum concentration measured was 12 μg/L [36]. The threshold concentration for reproductive toxicity of 1,300 μg/L determined in the present study suggests that this compound poses no significant risk of toxicity to daphnids from anti-ecdysteroidal activity at levels expected to occur in the environment.

*Acknowledgement*—This work was supported by U.S. Environmental Protection Agency grants R82935801 and RD83130001 to G.A. LeBlanc, National Institute of Environmental Health Sciences training grant ES007046 to C.V. Rider, and Korean Ministry of Science and Technology grant R05-2004-000-10180-0 to G.S. Hwang.

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