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THE IN VIVO ESTROGENIC AND IN VITRO ANTI-ESTROGENIC ACTIVITY OF PERMETHRIN AND BIFENTHRIN

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Abstract—Pyrethroids are highly toxic to fish at parts per billion or parts per trillion concentrations. Their intended mechanism is prolonged sodium channel opening, but recent studies reveal that pyrethroids such as permethrin and bifenthrin also have endocrine activity. Additionally, metabolites may have greater endocrine activity than parent compounds. The authors evaluated the in vivo concentration-dependent ability of bifenthrin and permethrin to induce choriogenin (an estrogen-responsive protein) in *Menidia beryllina*, a fish species known to reside in pyrethroid-contaminated aquatic habitats. The authors then compared the in vivo response with an in vitro assay—chemical activated luciferase gene expression (CALUX). Juvenile *M. beryllina* exposed to bifenthrin (1, 10, 100 ng/L), permethrin (0.1, 1, 10 μg/L), and ethinylestradiol (1, 10, 50 ng/L) had significantly higher ng/mL choriogenin (Chg) measured in whole body homogenate than controls. Though Chg expression in fish exposed to ethinylestradiol (EE2) exhibited a traditional sigmoidal concentration response, curves fit to Chg expressed in fish exposed to pyrethroids suggest a unimodal response, decreasing slightly as concentration increases. Whereas the in vivo response indicated that bifenthrin and permethrin or their metabolites act as estrogen agonists, the CALUX assay demonstrated estrogen antagonism by the pyrethroids. The results, supported by evidence from previous studies, suggest that bifenthrin and permethrin, or their metabolites, appear to act as estrogen receptor (ER) agonists in vivo, and that the unmetabolized pyrethroids, particularly bifenthrin, act as an ER antagonists in cultured mammalian cells. Environ. Toxicol. Chem. 2012;31:2848–2855. © 2012 SETAC

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INTRODUCTION

Pyrethroid pesticide use has undergone a considerable increase as organophosphate pesticides are phased out because of concerns regarding mammalian toxicity [1,2]. Pyrethroids, which are not acutely toxic to mammals at concentrations applied or found in the environment, are highly toxic to fish and aquatic invertebrates at parts per billion (ppb) or parts per trillion (pptr) levels [3,4]. Although pyrethroid bioavailability may be reduced in aquatic environments because these compounds are lipophilic and tend to bind to sediments instead of remaining dissolved, pyrethroids may remain in the water column for days to weeks after introduction [5,6], and they are soluble enough to produce biological and toxic effects [3].

Pyrethroids disrupt the nervous system via prolongation of the opening of voltage-dependent ion channels, the consequences of which are convulsions, paralysis, and eventual death [2,3]. Sublethal neurotoxic effects include impaired swimming ability in both fish and invertebrates [7] and a reduced ability to avoid predators [8]. In addition to effects produced by the intended mechanism, recent results from in vitro assays show that some pyrethroids can act as estrogens, anti-estrogens, or anti-androgens [9,10]. Pyrethroid metabolites are reported to have even greater endocrine activity than their parent structures in vitro [11–13]. Pyrethroids also have considerable endocrine activity in vivo, particularly bifenthrin, permethrin, and especially permethrin metabolites. These can induce estrogen-dependent egg proteins in male fish [13,14]. Notably, permethrin metabolites induced relatively higher expression of such proteins than the parent compound in a recent study [13].

Permethrin and bifenthrin are two of the most frequently detected pyrethroid pesticides in aquatic ecosystems [15–18]. Both are used in agriculture, but the more toxic bifenthrin is also increasingly used for landscaping and structural pest control under the trade name Talstar [16]. Because of their increasing presence in aquatic ecosystems and their potential to cause endocrine disruption, we describe studies examining the biochemical mechanisms and endocrine-disrupting effects of low pyrethroid concentrations using a combination of in vitro and in vivo approaches.

The first objective of the present study was to evaluate the concentration–response to bifenthrin and permethrin in vivo in *Menidia beryllina*, a fish species known to reside in pyrethroid-contaminated aquatic habitats. Our second objective was to use a biomarker of estrogenic endocrine disruption that is potentially more sensitive than vitellogenin [19], the estrogen-dependent egg yolk protein typically measured after exposure to suspected endocrine-disrupting compounds (EDCs) [20]. Although vitellogenin is a reliable marker of estrogenic endocrine disruption, we instead measured expression of choriogenin (Chg), an estrogen-dependent egg coat protein, using a *Menidia*-specific antibody [21]. Choriogenin, the precursor to the chorion or zona radiata, has been demonstrated to be more sensitive than vitellogenin [19,22] and was shown recently to have increased messenger RNA (mRNA) expression in larval fish after bifenthrin exposure [23].

All Supplemental Data may be found in the online version of this article.

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The third objective was to compare the response in fish with that in an estrogen-responsive cell bioassay (i.e., chemical-activated luciferase gene expression [CALUX]). In contrast to the widely utilized yeast estrogen screen (YES), CALUX has increased sensitivity because it has natively expressed estrogen receptors (ERs) and expresses mRNA for both ER alpha and beta [24]. To the best of our knowledge, this is the first study to evaluate the concentration–response of an estrogen-responsive protein in fish to pyrethroid pesticides and is the first study to use CALUX to evaluate the estrogenic activity of pyrethroid pesticides.

MATERIALS AND METHODS

Bioassay

We conducted a 14-d static aqueous exposure (with daily water renewal) using 65- to 70-d-old juvenile *M. beryllina* (Aquatic Biosystems). Fish were exposed to three concentrations of bifenthrin (1, 10, 100 ng/L), permethrin (0.1, 1, 10 μg/L), or ethinylestradiol (EE2; positive control: 1, 10, 50 ng/L) spiked into laboratory control water. Control water consisted of distilled water and filtered sea water from Bodega Bay, CA, USA (5 μm) mixed to achieve a salinity of 5 ± 1 parts per thousand, which is within the range of salinity in *M. beryllina* habitat [25]. Stock solutions of 1 μg/L for bifenthrin, permethrin, and EE2 were made in methanol (MeOH) for all concentrations except 1 and 10 μg/L permethrin, for which a stock solution of 10 μg/mL was used. Methanol at a concentration equal to the highest amount used in bifenthrin and permethrin treatments (0.01%) was spiked into laboratory control water as the negative control. This concentration of methanol is approved for use in toxicity testing with fish [26]; therefore, a non-methanol control was not included. Different ranges of concentrations (μg/L vs ng/L) were used because bifenthrin and permethrin differ substantially in toxicity, the former being more toxic. The highest concentration used for permethrin (10 μg/L) was below the 96-h median lethal concentration (LC50) of 27.5 μg/L for larval *M. beryllina* [2], and this concentration has already been shown to induce estrogen-responsive proteins (vitellogenin) in a fish closely related to *M. beryllina,* Japanese medaka (*Oryzias latipes*) [13]. The highest concentration for bifenthrin (100 ng/L) was well below the 144-h LC50 in larval bluegill sunfish (*Lepomis macrochirus*—350 ng/L) and two orders of magnitude below the 96-h LC50 in larval sheepshead minnow (*Cyprinodon variegatus*—17.8 μg/L), two species that have higher sensitivity to permethrin than *M. beryllina* [2]. The highest concentrations of bifenthrin, permethrin, and EE2 were intended to induce maximal expression of Chg without causing significant mortality, and the lowest concentrations were intended to mimic environmentally realistic exposures. Mean survival in all treatments after 14 d was $\geq$80%, conforming to U.S. Environmental Protection Agency (U.S. EPA) chronic toxicity testing standards [27], with the exception of the 10 μg/L permethrin treatment in which mean survival was 70%. Permethrin (purity, 99%) and ethinylestradiol (purity, 99%) were obtained from Sigma Aldrich, and bifenthrin (purity, 99%) from the U.S. Geological Survey (USGS) Analytical Chemistry Laboratory (Sacramento, CA). Pyrethroids were 50/50 mixtures of isomers.

Fish were maintained in 3-L glass jars with 1 L test water in each (10 fish/jar). The experiment was maintained at a 14-h/10-h light/dark cycle and temperature was controlled at 21 ± 2°C. Each jar was aerated, and dissolved oxygen, ammonia, and pH were measured daily before water changes. Fish were fed live or frozen *Artemia* nauplii each day at least 1 h before water change. For the bioassays, four replicates were present per treatment (except the positive control, which had two replicates). Each of these replicates consisted of 10 individual juvenile fish (65–70 d old at test initiation).

At test termination, fish were anesthetized on ice, immediately snap-frozen with liquid nitrogen, and stored at −80°C. Surviving whole fish (6–10 per replicate) were pooled and pulverized in approximately 5 ml liquid nitrogen with a ceramic mortar and pestle. Once ground to a powder, ice-cold 50 mM Tris-HCl homogenization buffer with protease inhibitor (Roche COmplete Mini) was added at a ratio of 1 ml buffer:2 g tissue and further homogenized with a Fisher Scientific Tissuemiser (ThermoFisher Scientific) for 60 s. The homogenate was centrifuged for 1 h at 20,800 *g* at 4°C, and the resulting supernatant was removed and recentrifuged at 14,850 *g* for 15 min to ensure removal of all particulates. The final supernatant was stored immediately at −80°C and the protein concentration of each sample quantitated using the BCA protein assay (Pierce).

Enzyme-linked immunosorbent assay

A *Menidia* polyclonal antibody, produced and optimized from *Menidia* chorion, was used to measure the relative amount of Chg expressed in whole body homogenate via an indirect enzyme-linked immunosorbent assay (ELISA), as previously described [21]. This methodology allows for accurate quantification of the amount of Chg in each sample relative to *Menidia* chorion levels expressed in the absence or presence of EE2.

Chemical-activated luciferase gene expression assay

The estrogen receptor–based CALUX mammalian cell bioassay uses a human ovarian carcinoma (BG-1) cell line, which has been stably transfected with an estrogen-responsive luciferase reporter plasmid. This CALUX cell line responds to estrogenic chemicals with the induction of expression of firefly luciferase. The CALUX bioassay has advantages over the yeast-based YES bioassay commonly used to detect estrogenic chemicals in that it expresses mRNA for ER isoforms alpha and beta [24], in contrast to the YES assay that is usually only transfected with ER alpha. The CALUX bioassay was used to determine the concentration-dependent agonist (pesticide alone) and antagonist (pesticide in presence of estradiol) effects of pyrethroids used in these studies and chemical exposure and luciferase analyses were carried out as described in detail [28] (see Supplemental Data). The source of 17β estradiol used as a positive control in all CALUX bioassays was from Sigma Chemical.

Pyrethroid extraction and analysis

Pyrethroid-spiked water samples, both time zero and 24 h, were sent to the USGS Analytical Laboratory for confirmatory chemistry. Water samples were filtered through 0.7-mm glass fiber filters, extracted onto Oasis HLB solid-phase extraction cartridges, and analyzed by gas chromatography electron ionization mass spectrometry in selective ion monitoring mode. Methods were performed as described in Hladik et al. 2008 [29] (see Supplemental Data). All concentrations of bifenthrin and permethrin had acceptable recoveries (80–110%).

Data analysis

Choriogenin ELISA data. Enzyme-linked immunosorbent assay data were first quantified by comparison of absorbance values with a chorion standard curve to obtain Chg equivalents. Equivalents were then divided by the protein concentration per

well to normalize the data as described in detail previously [21]. Then two types of statistical analyses were performed on Chg data: a traditional analysis of variance (ANOVA) followed by a Tukey honestly significant difference (HSD) test to detect whether differences were present between Chg expression induced by different compounds (i.e., ethinylestradiol vs bifenthrin), and nonlinear regression to fit concentration–response curves for each compound.

Because relationships between continuous variables, such as a concentration–response curve, cannot be properly described using ANOVA [30], we used nonlinear regression to evaluate the concentration–response for each compound (see Supplemental Data). The patterns of Chg expression in our experimental treatments did not appear to correspond to the sigmoidal concentration–response curve one would expect for increasing concentrations of a toxic compound. Therefore, we used a model selection approach to choose the concentration–response curve that was best supported by our data, as done in a previous toxicological study with three concentrations each of the pyrethroids permethrin and cyfluthrin [4]. In general, concentration–response curves with more parameters (i.e., greater complexity) will afford a better fit to data. However, models with more parameters run the risk of overfitting: they may fit the random variation in a small data set well but be unsuitable for predicting the underlying relationship or trend. We avoided overfitting by using information theory criteria to identify the most parsimonious model; that is, the model that affords the best fit with the fewest parameters [31]. We fit two different curves to each Chg dataset: a sigmoidal curve (three-parameter logistic model) and a unimodal curve (a modified three-parameter logistic model [32]). Curves were fit by nonlinear least squares using the trust-region reflective algorithm in Matlab 7.11 (The Mathworks). The most parsimonious model was determined using Akaike's Information Criterion corrected for small sample sizes (AIC_c), a method that is specifically intended to avoid overfitting small datasets [31]. All curves were fit to $\log(x + 0.01)$ transformed concentrations data to avoid the problem of taking the log of concentration 0 (MeOH control).

CALUX. For the CALUX bioassays, an individual *t* test was used to determine whether treatments were significantly different from the relevant positive control (estradiol), expressed as 100% cellular response. Resulting *p* values were Bonferroni corrected to account for multiple comparisons. Differences among treatments in estrogen equivalents were tested using an ANOVA followed by a Tukey HSD test. All ANOVAs were performed with the statistical software R 2.11.1 [33].

RESULTS

Differences between treatments

Juvenile *M. beryllina* exposed to all three concentrations of bifenthrin (1, 10, 100 ng/L), permethrin (0.1, 1, 10 μg/L), and EE2 (1, 10, 50 ng/L) had significantly higher relative ng/ml Chg expressed in whole-body homogenate than the methanol control (Fig. 1). None of the pyrethroid treatments were significantly different from one another when compared using ANOVA ($p > 0.05$); however, a trend toward an inverse correlation between response and increasing concentration with both permethrin and bifenthrin is apparent (Fig. 1). The results pertaining to this trend will be further explained in the *Concentration–Response* section. Average water quality parameters measured before daily renewal for temperature, pH, dissolved oxygen, and ammonia were 21.0 ± 0.5°C, 7.5 ± 0.2, 8.5 ± 0.6 mg/L, and 0.2 ± 0.1 mg/L respectively.

Permethrin and bifenthrin treatments were not significantly different from positive controls of 1 and 10 ng/L EE2 over a 100-fold concentration range, with the exception of 1 ng/L bifenthrin, which had a significantly higher relative Chg level than that produced by 1 ng/L EE2 ($p = 0.022$). However, all permethrin and bifenthrin concentrations trend toward having a higher relative expression of Chg than the 1 ng/L EE2 treatment, and a lower relative expression than the 10 ng/L EE2 treatment. A concentration-dependent increase in Chg levels was observed with EE2 exposure. The 1 ng/L EE2 treatment is significantly different from both 10 ng/L and 50 ng/L EE2. Whereas the 10 and 50 ng/L EE2 treatments were not significantly different, the trend (mean values) was progressively higher with increasing concentration.

Concentration–response

Concentration–response curves for ethinylestradiol, permethrin, and bifenthrin (Fig. 2) were generated using nonlinear least squares regression, and curve fit was evaluated using AICc ([31]; AIC adjusted for small sample sizes). As expected, a sigmoidal model had the most parsimonious fit to the EE2 response (Fig. 2), with a delta $AIC_c = 0$ (Supplemental Data). The curve does not reach an asymptote, likely because Chg induction may not have reached a maximum at our highest exposure of 50 ng/L.

In contrast to the results for EE2, the best (i.e., most parsimonious) concentration–response curve for induction of Chg by bifenthrin and permethrin is not sigmoidal, but a unimodal, or biphasic curve (Fig. 2). A unimodal model, in which the response peaks and then begins to decrease as concentration increases, has a more parsimonious fit to the data ($AICc = 0$) than a linear or sigmoidal model, despite having more parameters (delta $AICc = 0.3$ and 5.0, respectively).

CALUX assay

The CALUX assay did not detect ER agonism for any of the permethrin or bifenthrin concentrations tested (Fig. 3). In the ER antagonism assay, however, an initial bifenthrin concentration-dependent decrease in the ability of E2 to induce ER-dependent reporter gene activity (1–100 ng/L bifenthrin) was observed, with a recovery of estrogenic activity at bifenthrin concentrations greater than 100 ng/L (Fig. 3). In contrast, although some permethrin-dependent reduction in estrogenic activity was observed (~30–40% of maximal estradiol activity), no concentration dependence of this inhibitory effect was seen.

Dissolved pyrethroid concentrations

As expected, a decrease occurred in the dissolved pyrethroid concentrations over a 24-h period. Whereas initial (day 0) dissolved concentrations were within 90–111% of nominal concentrations, concentrations measured after approximately 24 h, before daily bioassay water changes, had decreased to 64 to 73% of nominal (Table 1). This decrease in pyrethroid concentration after 24 h is likely attributable to adsorption of bifenthrin and permethrin to walls of the glass jars used [34,35]. Levels of bifenthrin and permethrin were not measured in fish tissue, because all of the samples were required for protein analysis.

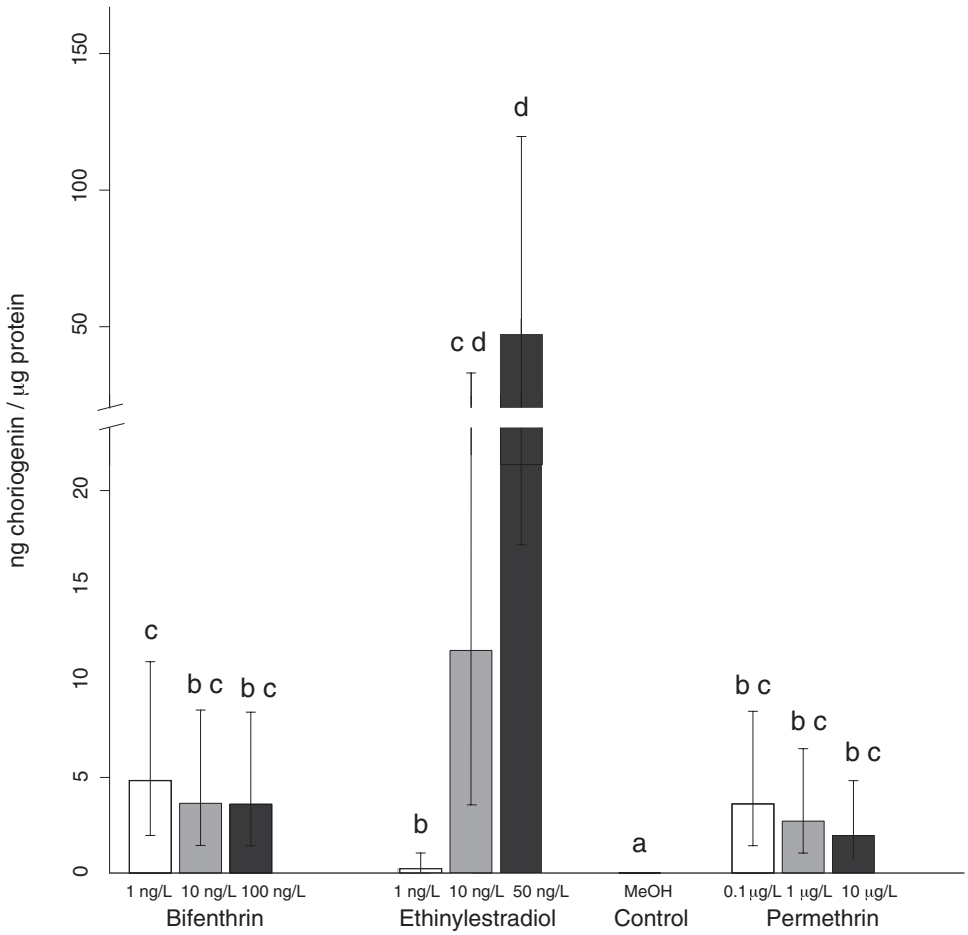

Fig. 1. Effect of bifenthrin, permethrin, and ethinylestradiol on choriogenin expression in juvenile *Menidia beryllina*. Fish were exposed to the indicated concentration of each compound in water (with daily water changes) for 14 d followed by determination of choriogenin levels. Values represent the mean ± 95% confidence limits of whole body homogenate from four pooled replicates of 6 to 10 fish each (bifenthrin, permethrin) and two pooled replicates of 10 fish each (ethinylestradiol) as determined by analysis of variance (ANOVA). Significant differences between treatments were determined via a Tukey test. Treatments that were not significantly different ($p > 0.05$) are indicated by the same letter, and treatments that are significantly different from each other ($p < 0.05$) are indicated by different letters.

DISCUSSION

The first objective of the present study was to evaluate the concentration–response to bifenthrin and permethrin in vivo, in a fish species known to reside in pyrethroid-contaminated aquatic habitats. To achieve this goal, we evaluated the effects of a range of bifenthrin and permethrin concentrations, some near or below those found recently in storm drain runoff (bifenthrin 73 ng/L and permethrin 66.1 ng/L [16]) on *M. beryllina* (inland silverside) and close to the low nanogram per liter concentrations frequently detected in the environment [2]. Our second objective was to use a biomarker of estrogenic endocrine disruption that is potentially more sensitive than vitellogenin [19], the estrogen-dependent egg yolk protein typically measured after exposure to suspected EDCs [20]. In lieu of vitellogenin, we used a *Menidia*-specific antibody to detect Chg, an estrogen-dependent egg coat protein via indirect ELISA [21]. The third objective was to compare fish response to an in vitro response by employing the CALUX assay. With the CALUX assay, it was possible to examine the potential for additivity, antagonism, or synergism of pyrethroid pesticides with endogenous estrogen. To our knowledge, this is first study to test the endocrine concentration–response of bifenthrin and permethrin at or near environmentally relevant concentrations. Additionally, this is the first study to use the CALUX assay, which expresses mRNA for both ER alpha and beta [24] to evaluate pyrethroid endocrine activity.

Comparability of response with other estuarine fish species is afforded by the use of *Menidia* [21]. Silversides are ubiquitous in and easily collected from estuarine, brackish, and freshwater habitats [25], they are exposed to runoff containing pyrethroids throughout North America and in the Sacramento-San Joaquin Delta, and are commercially available for laboratory studies. Furthermore, the inland silverside has a higher published permethrin LC50 than many other fish species [2]. Though this may contribute to increased survival of silversides, it potentially renders them more susceptible to pyrethroid-induced endocrine disruption. Early life exposure to these low concentrations of pyrethroids may be particularly damaging to silverside populations because the sex ratio of *Menidia* spp. has been shown to be susceptible to estrogen exposure during the larval period [36]. Notably, the present study is the first to look at protein-level endocrine-disrupting effects of pyrethroids on juvenile fishes, which are more sensitive to such environmental EDC perturbations than adults [14] and are more likely to experience long-term developmental changes such as reduced fecundity or intersex when exposed to EDCs [37].

Responses in fish

The ability of single concentrations of permethrin and bifenthrin to induce egg yolk expression (vitellogenin) in male or juvenile fish, indirectly indicating ER binding or activation of the estrogen response element, has been demonstrated in a

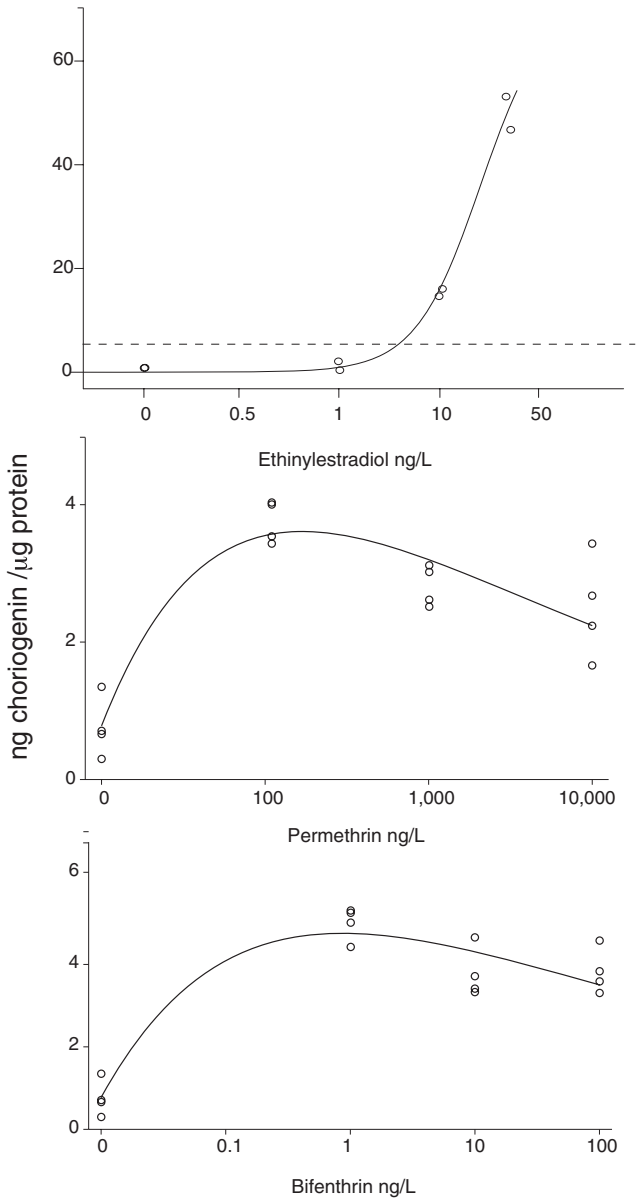

Fig. 2. Choriogenin concentration–response of juvenile *Menidia beryllina* to ethinylestradiol, bifenthrin, and permethrin. Most parsimonious curve fit to concentration–response data (sigmoidal or unimodal) using nonlinear regression. Each data point represents the combined whole body homogenate of 6 to 10 juvenile *M. beryllina* exposed for 14 d to the corresponding concentration. The horizontal dotted line across the top panel represents the maximum choriogenin response in pyrethroid-exposed fish.

number of other studies [13,38,39]. Although vitellogenin is a reliable marker of estrogenic endocrine disruption, we instead measured expression of Chg, using a *Menidia*-specific antibody [21]. Choriogenin, which is a precursor to zona radiata, is an egg coat protein demonstrated to be more sensitive than vitellogenin in previous studies [1,22,40], and was shown recently to have increased mRNA expression in larval fish after bifenthrin exposure [23]. Like vitellogenin, this protein is normally only induced by endogenous estrogen in mature females [40] but is expressed in males and juveniles exposed to exogenous estrogens or estrogenic EDCs [22,40].

The present study found that all concentrations (0.1, 1, 10 µg/L permethrin and 1, 10, 100 ng/L bifenthrin) significantly induced the expression of Chg in juvenile *M. beryllina* in comparison with methanol controls. Several of these concentrations are an order of magnitude lower than those shown in previous studies to induce estrogen-dependent mRNA or protein production. The concentrations used in the present study decreased substantially over the 24-h time course between water changes, indicating that fish were responding to decreasing concentrations compared with those present at the start of dosing each day (at the water change). Notably, we observed that 1 ng/L bifenthrin induced significantly greater Chg expression than 1 ng/L EE2. This is alarming, considering that EE2 is a potent ER agonist that has even greater estrogenicity than endogenous estradiol [41]. This finding indicates that bifenthrin is estrogenic at the pptr levels that are regularly detected in aquatic ecosystems and also argues for future testing of concentrations below 1 ng/L. Considering that the current method detection limit for bifenthrin is 0.5 ng/L, the ability to test and detect concentrations below this presents a challenge to both toxicologists and chemists.

Although the ANOVA showed no significant difference in Chg expression between pyrethroid concentrations, the best-fit curve for both pyrethroids suggests a biphasic or unimodal response. In contrast, EE2 displayed the expected sigmoidal response, previously confirmed by others [42]. Though the unimodal fit as evaluated by AICc is not as parsimonious for bifenthrin as it is for permethrin, the graphical trend is the same, suggesting that a unimodal curve is the best fit for both. The apparent difference in results between ANOVA and curve-fitting analyses is not surprising. ANOVA is designed to detect differences between categorical treatments (e.g., comparing bifenthrin with EE2 response), and so it is not ideal for detecting trends in data with a continuous predictor variable (e.g., increasing concentrations of same compound). It effectively loses information by not accounting for the ordered relationship among treatments. Additionally, ANOVA has less statistical power than a curve-fitting approach to detect trends [30]. As such, curve fitting is a more powerful approach to detect trends such as the shape of a concentration–response curve, although ANOVA remains useful for detecting differences among treatments that do not have an ordered relationship, as we show in Figure 1. Other studies have noted that some estrogenic compounds (i.e., bisphenol A) induce an inverted U-shaped or unimodal dose response because of low dose stimulation and higher dose inhibition, likely attributable to receptor-mediated responses that can saturate or vary depending on concentration [43–45]. Further studies are needed to determine whether the endocrine response to permethrin and bifenthrin is truly unimodal and to provide further information on the mechanisms underlying the trend observed here.

Responses in cell lines

Evidence for a difference in estrogenicity between the parent pyrethroid compounds and their metabolites is demonstrated by the results of the CALUX assay, which natively expresses the human estrogen receptor (in BG-1 cells). Using a cell line that expresses the human estrogen receptor is comparable to evaluating the fish ER response in vitro, because high sequence identity is seen between human and fish steroid receptors [46,47]. As has been demonstrated in other studies with permethrin [9,48], the CALUX assay results demonstrate that bifenthrin acts as an estrogen antagonist (Fig. 3). The antagonism of bifenthrin increases with increasing concentration to a maximum of 100 ng/L, whereas permethrin's antagonistic properties do not correlate with concentration tested. Thus, this inhibition is unlikely to result from a direct antagonistic effect

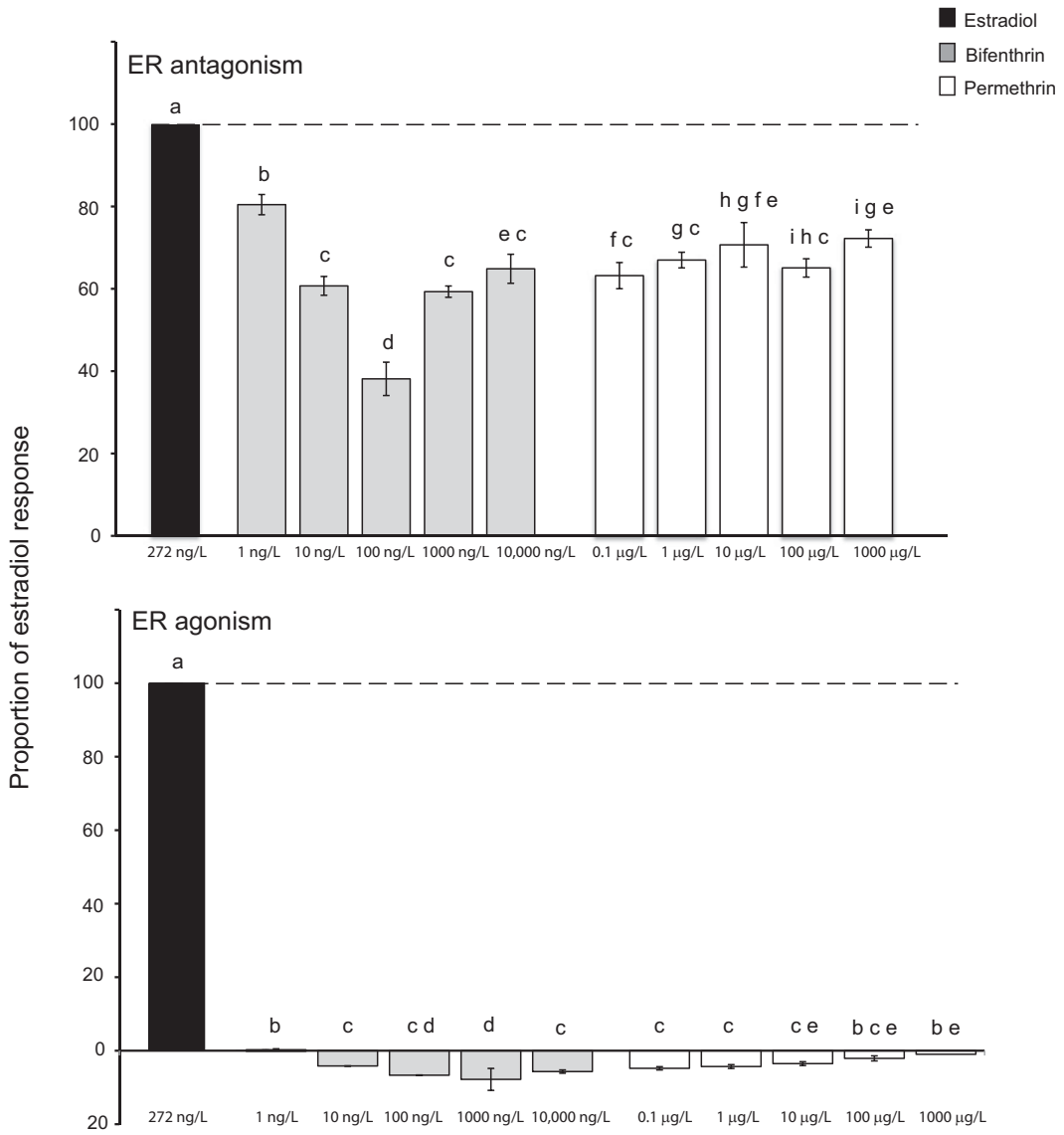

Fig. 3. Activation and inhibition of the estrogen receptors (ER) chemical activated luciferase gene expression (CALUX) by bifenthrin and permethrin. The CALUX ER agonist and antagonist activity are presented as a proportion of the estradiol (272 ng/L = 1 nM) positive control (100%). Error bars represent 95% confidence intervals. With ER antagonism ($n = 3$ replicate wells), values below 100% indicate the occurrence of antagonism. With ER agonist activity ($n = 3$ replicate wells), values below 20% of the estradiol control indicate that bifenthrin and permethrin are not ER agonists at that concentration. Significant difference of all treatments from E2 controls in both assays was determined via Bonferroni corrected individual t tests ($p < 0.05$) and is indicated with the letter "a." Tukey test results are represented by letter codes "b" through "i" above each bar. Treatments with different letters are significantly different from each other ($p < 0.05$); treatments sharing letter codes are not significantly different ($p > 0.05$).

Table 1. Chemical analysis of aqueous permethrin and bifenthrin concentrations[a]

Compound	Nominal concentration	Actual concentration (0 h)	Actual concentration (24 h)
Bifenthrin	1 ng/L	0.898 ng/L	0.733 ng/L
	10 ng/L	9.514 ng/L	6.890 ng/L
	100 ng/L	111 ng/L	71 ng/L
Permethrin	0.1 µg/L	0.092 µg/L	0.068 µg/L
	1 µg/L	1.05 µg/L	0.641 µg/L
	10 µg/L	9.12 µg/L	6.52 µg/L

[a] Concentrations of bifenthrin and permethrin were measured via gas chromatography–mass spectronomy in newly spiked laboratory control water and 24 h later in water that had been used for the *Menidia beryllina* 14-d bioassay.

of permethrin on the estrogen receptor or estrogen receptor–signaling pathway. This could indicate that toxicity is occurring via other mechanisms, with the higher concentrations of permethrin relative to bifenthrin affecting the cells' ability to respond.

On examination of other research findings along with ours, the lack of a consistent linear relationship between antagonism and concentration may be caused by bifenthrin and permethrin acting via different mechanisms at different concentrations or conditions. For example, whether permethrin blocks binding to the ER appears to depend on the cell line used [9,48]. Additionally, both bifenthrin and permethrin have been shown to activate expression of an estrogen-linked transcription factor in some studies [9,49], but permethrin did not in others [48,50]. Permethrin's action at the ER is clearly dependent on the combination of cell line, concentration used, and endpoint

examined, and considering the results of the present study bifenthrin appears to exhibit similar contradictory behavior.

Neither compound acted as an estrogen agonist in the CALUX assay at any concentration, a finding that is somewhat surprising considering results of previous research that indicate bifenthrin and permethrin operate as ER agonists via the classical nuclear receptor pathway [11,38]. The difference seen may be accounted for by use of the CALUX assay, rather than the YES assay, which is a yeast cell line commonly used for testing estrogenic endocrine activity to date. Notably, unless modified to do so, the YES assay does not discriminate well between agonists and antagonists [12], and the YES assay also does not express mRNA for both ER alpha and beta as the CALUX assay does [21], because it is normally only transfected with ER alpha [51]. As such, the mammalian cell CALUX assay may offer a more realistic assessment of receptor-dependent estrogenic or anti-estrogenic activity. Additionally, nearly all previous cell line studies conducted with pyrethroids used concentrations that were higher than those used in our study, by as much as an order of magnitude [11]. As mentioned earlier, the receptor-mediated responses evaluated here can be saturated or vary widely depending on concentration [45,52], which may explain the difference in endocrine activity between the low concentrations used in our study with higher concentrations used in previous in vitro studies.

CONCLUSION

Our experimental evidence indicates that permethrin and bifenthrin are estrogenic in vivo but anti-estrogenic in cells in vitro. The difference in results between the in vivo and in vitro assays used in the present study may be attributable to the lack of appropriate metabolism in the CALUX cell line. This finding is corroborated by a number of studies that show that pyrethroid metabolites are more estrogenic than their parent compounds [11–13].

Future studies evaluating the activity of pyrethroid metabolites in the CALUX assay and studies that elucidate the action of permethrin, bifenthrin, and their metabolites at each ER isoform (alpha, beta, gamma) are necessary. Furthermore, using an in vivo system to compare in vitro responses with when evaluating endocrine responses [53] and testing concentrations in the vicinity of those that have been detected in the environment are important considering our findings.

The estrogenic or anti-estrogenic activity exhibited by these compounds is likely to exacerbate their established toxicity in aquatic ecosystems. Furthermore, their ability to exert effects on the endocrine system of fishes at concentrations at or near those regularly detected in watersheds is cause for great concern, particularly with bifenthrin, especially when considering recent research that suggests changes in estrogen-dependent protein expression in fishes may precipitate population decline [54] and that the pyrethroid mixtures that occur in aquatic habitats likely have additive endocrine toxicity.

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