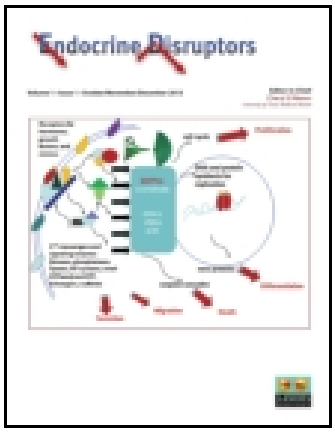

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Heather A Thorn[a], John E Quinn[b] & Alison M Roark[ab]
[a] Department of Biology; Hood College; Frederick, MD USA
[b] Department of Biology; Furman University; Greenville, SC USA
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Reproductive and developmental effects of tributyltin, bisphenol A, and 17 β-estradiol in pale anemones (*Aiptasia pallida*)

Heather A Thorn[1], John E Quinn[2], and Alison M Roark[1,2,*]

[1]Department of Biology; Hood College; Frederick, MD USA; [2]Department of Biology; Furman University; Greenville, SC USA

Keywords: Akaike's information criterion (AIC), Anthozoa, asexual reproduction, binomial-Poisson hurdle model, Cnidaria, development, estrogen receptor, estrogenic, generalized linear model, metazoa

Abbreviations: BPA, bisphenol A; E2, 17 β-estradiol; TBT, tributyltin.

The effects of exposure to estrogenic endocrine-disrupting chemicals in most clades of marine invertebrates are unknown. The purpose of this study was to determine if exposure to 3 such chemicals modulates asexual reproduction and development in pale anemones (*Aiptasia pallida*). Anemones (n = 18 in each group) were exposed for 21 days to one of 8 treatments: seawater alone, seawater containing vehicle, or seawater containing a low (environmentally relevant) or high dose of tributyltin (TBT), bisphenol A (BPA), or 17 β-estradiol (E2) dissolved in vehicle. The number of asexually generated pedal lacerates produced by each anemone and the number of days required for each lacerate to develop a stomodeum and tentacles were recorded. At the end of the study, parent anemones were homogenized, and total protein content (as a proxy for body size) was quantified by Bradford assay. The roles of chemical treatment and parent anemone size in determining lacerate production were evaluated with binomial-Poisson hurdle models, and their roles in determining development rate were evaluated with generalized linear models. Application of model selection criteria suggested that exposure to E2 (at 45 ng/L) but not to TBT or BPA was associated with increased pedal lacerate production. Neither low nor high doses of any chemical tested affected the number of days required for lacerates to develop into juveniles. Although cnidarians are not thought to express genes homologous to vertebrate estrogen receptors, evidence from this and other studies suggests that estrogens, at least at high doses, are bioactive in these basal metazoans.

Introduction

Endocrine-disrupting chemicals (EDCs) are natural or man-made substances that interfere with the synthesis, secretion, transport, activity, or clearance of hormones in the body,[1] thereby disrupting homeostasis, development, reproduction, and/or behavior of animals. Many EDCs function as estrogen receptor (ER) agonists and can have profound effects on reproductive function. For example, exposure to estrogenic chemicals is associated with polycystic ovary syndrome, decreased sperm count, and prostate cancer in humans and with a number of reproductive abnormalities in other animals.[2] Although estrogenic EDCs have been well studied in vertebrate animals, their effects in many clades of organisms, particularly marine invertebrates, are largely unknown.

Marine animals may be especially susceptible to EDC exposure, as both sewage and wastewater treatment plant (WWTP) effluent contain a variety of estrogenic EDCs and serve as a continuous source of these chemicals into aquatic ecosystems.[2,3] The purpose of the present study was to investigate whether reproduction of pale anemones (*Aiptasia pallida*) could be modulated by exposure to 17 β-estradiol (E2) or either of 2 estrogenic EDCs (bisphenol A and tributyltin). E2 is the predominant endogenous estrogen in vertebrates, although it can also be found in invertebrates including cnidarians.[4,5] Bisphenol A (BPA) is a component of many plastics and resins and is a known agonist of the estrogen receptor.[6] Until recently, tributyltin (TBT) was included in anti-fouling paints used on boat hulls. In mammalian cells, TBT demonstrates both adipogenic activity at low doses and estrogenic activity at high doses,[7] and exposure to TBT is associated with shell calcification anomalies in oysters as well as imposex and sterility in marine gastropods.[8]

Aiptasia pallida anemones have been proposed as a model organism for biomonitoring of polluted environments.[9] These

*Correspondence to: Alison M Roark; Email: alison.roark@furman.edu
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cnidarians reproduce both sexually via broadcast spawning and asexually via pedal laceration, in which cloned offspring bud from the pedal disc of the parent.[10] *Aiptasia pallida* anemones are widely distributed throughout the Atlantic and Gulf coasts of the southern United States from North Carolina to Texas and in the Caribbean Sea.[11] They reside on rocks, mangrove roots, coral, and other hard substrates in near shore, shallow coastal waters. Given their proximity to human population centers, these habitats are likely influenced by estrogenic EDCs from point and non-point sources including WWTPs, sewage, and industrial facilities.

To date, only a handful of studies have evaluated the potential reproductive effects of estrogenic EDCs in cnidarians. However, these studies suggest that estrogens and other estrogenic chemicals are bioactive in these animals. For example, pore coral (*Montipora capitata*) exposed to estradiol demonstrated reduced production of egg-sperm bundles, and finger coral (*Porites compressa*) exposed to estrone grew more slowly than controls.[12] In freshwater *Hydra oligactis*, BPA (1 mg/L) suppressed sexual reproduction while simultaneously stimulating asexual reproduction.[13] Although exposure to TBT is associated with expulsion of algal symbionts in *A. pallida*,[14] the effects of TBT on cnidarian reproduction are unknown.

In this study, adult *A. pallida* anemones were maintained individually and exposed for 21 days to low and high concentrations of E2, BPA, and TBT (**Table 1**). The low doses that we used reflect environmentally relevant concentrations of each chemical. For example, in near shore environments, E2 has been detected at concentrations as high as 1.8 ng/L.[15] Concentrations of BPA in the open ocean are unknown, but reported concentrations of BPA in freshwater samples are typically 21 μg/L or less.[16] Prior to a 2008 global ban on the use of TBT in marine paints, TBT was measured at levels as high as 460 ng/L in ports and marinas and at concentrations of 1 to 10 ng/L in coastal waters.[17] Thus, we chose 1.8 ng/L as our low concentration of E2, 21 μg/L as our low concentration of BPA, and 25 ng/L as our low concentration of TBT.

The effects of these estrogenic chemicals on asexual reproduction were quantified by counting the number of asexually produced offspring (pedal lacerates) formed during the 3 week study. We also quantified the development rate of lacerates by counting the number of days required for pedal lacerates to develop both an esophagus (stomodeum) and tentacles. To our knowledge, this study is the first to examine the effects of estrogenic EDCs in anemones.

Table 1 Treatment groups, sample sizes, and concentrations used during the 21-day exposure period. Abbreviations: BPA = bisphenol A, TBT = tributyltin

		Concentration	
Treatment	***n***	**Low**	**High**
17 β-estradiol	18	1.8 ng/L	45 ng/L
BPA	18	21 μg/L	525 μg/L
TBT	18	25 ng/L	625 ng/L
Solvent Control	18	20 mL/L	—
Seawater Control	18	—	—

Results

Measured lacerate production, lacerate development time, and protein content of parental anemones are presented in **Table 2**. Mixed binomial-Poisson hurdle models and multi-model inference were used to determine whether lacerate production was predicted by protein content (as a proxy for anemone size) and/or treatment. The top model ($\Delta AIC < 2$) comparing seawater and solvent controls included treatment, protein content, and the interaction between treatment and protein content as predictors of lacerate production (**Table 3**), suggesting that our 2 control groups were not comparable. Thus, we only used data from the solvent controls (not from the seawater controls) in subsequent analyses.

The top models ($\Delta AIC < 2$) for tributyltin and BPA did not include treatment but did include protein content as a predictor of lacerate production (**Tables 4 and 5**). However, for 17 β-estradiol, the top model included the interaction between protein content and treatment (**Table 6A**). Parameter estimates for the binomial hurdle overlapped zero (**Table 6B**), suggesting that the probability of producing lacerates was not different between groups. However, the confidence intervals for the parameter estimates of the Poisson component that predicts lacerate abundance did not overlap zero (**Table 6C**), indicating that the number of lacerates produced differed between control and high estradiol-treated anemones. Specifically, anemones treated with the high dose of 17 β-estradiol had greater estimated lacerate production than the solvent control across protein levels (**Table 6C**, **Fig. 1**). The confidence interval for the parameter estimate of anemones treated with the low dose of estradiol overlapped zero, constraining our ability to predict lacerate abundance in these anemones.

A generalized linear model with a Gaussian distribution was used to test whether the age at which lacerates reached the juvenile stage was predicted by protein content and/or treatment. For this analysis, the null model was the top model ($\Delta AIC < 2$) for all treatments. Thus, treatment did not appear to affect the rate of development to the juvenile stage.

Discussion

In this study, we evaluated the effects of chemicals with known estrogenic activity on asexual reproduction and development in the pale anemone, *Aiptasia pallida*. In particular, we combined controlled lab experiments with multi-model inference to test if exposure to 17 β-estradiol (E2), bisphenol A (BPA), or tributyltin (TBT) modulated 3 different reproductive or developmental outcomes: the propensity to reproduce, the number of lacerates produced among those anemones that reproduced, and the rate of lacerate development.

As an alternative to comparing means, we used mixed generalized linear models due to the structure of our data.[18] Rather than

Table 2 Number of anemones that produced lacerates and ranges and medians of non-zero number of lacerates produced, average lacerate development time, and protein content of anemones treated for 21 days with seawater, solvent (2% acetone), or low or high doses of tributyltin (TBT), bisphenol A (BPA), or 17 β-estradiol (E2). Low and high doses are defined in **Table 1**

		Number of lacerates produced		Average lacerate development time (days)		Protein content of parent anemone (mg)	
Treatment	**Number of anemones that produced lacerates**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**
Seawater Control	5	1–11	1	5.0–5.6	5.5	0.96–5.35	2.05
Solvent Control	7	1–9	3	2.0–7.0	5.5	1.28–5.88	2.30
Low TBT	9	1–16	2	3.0–7.0	5.7	1.18–7.99	1.85
High TBT	5	1–12	3	1.1–10.0	5.2	0.97–5.26	2.14
Low BPA	9	1–10	3	3.0–7.0	5.0	1.00–5.09	2.27
High BPA	10	1–11	3	2.0–7.0	4.8	1.46–6.60	2.75
Low E2	10	1–6	2	1.5–8.0	6.0	0.97–6.84	1.93
High E2	12	1–17	3	0.5–7.0	5.0	1.73–7.85	2.29

forcing our data into a statistical framework that did not match the structure and distribution of our data (e.g., log-transforming count data[19]), we adopted modern statistical techniques that allow for analysis of data from binomial, Poisson, and other non-Gaussian distributions. Specifically, we used mixed binomial-Poisson hurdle models to account for the zero-inflated structure of our data. Hurdle models allow analyses of data sets containing multiple zeros (e.g., for the number of lacerates produced). The hurdle function constrains subsequent analysis of lacerate production to only those anemones that produced lacerates. The binomial hurdle of these models reflects whether or not anemones produced lacerates, and the Poisson component provides an estimate of the number of lacerates produced by those anemones that reproduced, avoiding zero inflation of the estimates.

We used AIC model selection and multi-model inference for several reasons. First, unlike traditional null hypothesis testing, AIC model selection is grounded in maximum likelihood estimation, a framework more suitable for modern statistical techniques. Additionally, multi-model inference allows for more robust parameter estimates by incorporating uncertainty from all appropriate models. Lastly, using multi-model inference avoids the use of arbitrary probability thresholds in determining statistical significance.[20]

Using this statistical approach, we first determined the parameter estimates for hurdle models that included treatment, anemone protein content, and/or the interaction between treatment and protein content. The top model predicting each dependent variable was the one with the lowest Akaike's Information Criterion (AIC) value of all tested models. We found that treatment did not affect whether or not anemones produced lacerates, regardless of the concentration or identity of the chemical tested. However, anemones exposed to vehicle (acetone) in seawater produced a different number of lacerates across a range of anemone sizes compared to anemones exposed to seawater alone. For this reason, anemones treated with each of the 3 estrogenic chemicals (all of which were dissolved first in vehicle and then in seawater) were only compared to anemones treated with vehicle.

We found that exposure to tributyltin at either low or high doses does not appear to modulate the number of lacerates produced relative to solvent controls. However, across all treatments of tributyltin and controls, larger anemones (with higher protein content) were more likely to produce more lacerates than smaller anemones (with lower protein content). Exposure to bisphenol A also had no effect on the number of lacerates produced relative to solvent controls.

Table 3 AIC model selection results and parameter estimates from binomial-Poisson hurdle models that predict lacerate production of anemones treated with either solvent (2% acetone) or seawater. A) Model selection results are listed in order of increasing AIC values, with the top model (ΔAIC<2) highlighted in bold. Parameter estimates for the B) binomial hurdle and C) Poisson count estimates in the top model are listed, with non-zero estimates (based on 95% confidence intervals) indicated by bullets (•). The binomial hurdle model results predict whether or not anemones will produce lacerates, whereas the Poisson count model results predict the number of lacerates produced among anemones that reproduced. Abbreviations: AIC = Akaike's Information Criterion, k = number of parameters, cum. = cumulative, * = both main effects and interaction were tested, : = only interaction was tested, + = only main effects were tested

A. Model selection results							B. Binomial hurdle model		C. Poisson count model	
Model	**k**	**AIC**	**ΔAIC**	**AIC weight**	**cum. AIC weight**	**Top Model Parameters**	**Estimate**	**Std. Error**	**Estimate**	**Std. Error**
protein*treatment	**8**	**102.37**	**0.00**	**0.86**	**0.86**	intercept	−0.71	1.14	−2.24	1.33
Protein	4	106.87	4.50	0.09	0.95	protein	−0.10	0.41	0.97•	0.29
protein:treatment	6	109.58	7.20	0.02	0.98	treatment	0.19	1.62	3.37•	1.38
protein+treatment	6	110.05	7.68	0.02	1.00	protein:treatment	0.13	0.58	−0.86•	0.32
Null	2	113.34	10.97	0.00	1.00					
Treatment	4	116.19	13.81	0.00	1.00					

Table 4 AIC model selection results and parameter estimates from binomial-Poisson hurdle models that predict lacerate production of anemones treated with either solvent (2% acetone) or low or high concentrations of tributyltin. A) Model selection results are listed in order of increasing AIC values, with the top model (ΔAIC<2) highlighted in bold. Parameter estimates for the B) binomial hurdle and C) Poisson count estimates in the top model are listed, with non-zero estimates (based on 95% confidence intervals) indicated by bullets (•). The binomial hurdle model results predict whether or not anemones will produce lacerates, whereas the Poisson count model results predict the number of lacerates produced among anemones that reproduced. Abbreviations: AIC = Akaike's Information Criterion, k = number of parameters, cum. = cumulative, * = both main effects and interaction were tested, : = only interaction was tested, + = only main effects were tested

A. Model selection results							B. Binomial hurdle model		C. Poisson count model	
Model	**k**	**AIC**	**ΔAIC**	**AIC weight**	**cum. AIC weight**	**Top Model Parameters**	**Estimate**	**Std. Error**	**Estimate**	**Std. Error**
protein	**4**	**168.86**	**0.00**	**0.65**	**0.65**	intercept	−1.33•	0.58	0.46•	0.23
protein+treatment	8	171.46	2.60	0.18	0.83	protein	0.33	0.19	0.27•	0.04
protein:treatment	8	172.64	3.78	0.10	0.93					
protein*treatment	12	173.25	4.38	0.07	1.00					
null	2	205.25	36.39	0.00	1.00					
treatment	6	211.21	42.35	0.00	1.00					

Conversely, and despite moderate sample sizes and a short exposure window, our study demonstrated a role for 17 β-estradiol in modulating the extent of asexual reproduction. Specifically, anemones treated with high concentrations of E2 produced more lacerates than solvent controls, with the magnitude of this difference increasing as the protein content of the parent anemone increased (**Fig. 1**).

Our data indicate that, at least under certain conditions, the rate of asexual reproduction is positively correlated with body size (as measured by protein content) in *Aiptasia pallida*. The size dependence of pedal laceration rate has been reported in other species of clonally reproducing anemones, including *Anthopleura elegantissima*[21] and *Metridium senile*.[22,23] Although the mechanism of induction of pedal laceration is unknown, it has been suggested[24] that tissue stretching, which occurs as an anemone elongates, may stimulate asexual reproduction in anemones. Other stimuli, including substrate instability[23] and starvation,[10] both of which may be associated with elongation of the body column, have also been shown to stimulate pedal laceration rate.

These results also provide additional support for a small but growing body of evidence that steroids are bioactive in cnidarians. For example, E2, estrone, progesterone, and testosterone have all been detected in tissues of scleractinian corals at levels that vary across seasons,[4,5,25,26] and E2 is released from these animals during mass spawning events.[5,26,27] It is not clear if these hormones are endogenously produced and regulated or if corals sequester exogenous steroids found in seawater. While several studies[5,28] have reported that corals express aromatase, other authors[29] found evidence to the contrary. At the very least, cnidarians are capable of metabolizing steroids, as genes in the short chain dehydrogenase/reductase family have been identified in corals,[30] and activity of steroidogenic enzymes such as 5 α-reductase, 3 β-hydroxysteroid dehydrogenase, and 17 β-hydroxysteroid dehydrogenase has been observed in corals.[5,25,31]

The role(s) and mechanism(s) of action of steroids in cnidarians are also unclear, as steroid receptors have not yet been identified in these animals.[32] Despite the apparent lack of a receptor homologous to vertebrate estrogen receptors, exposure to 17 β-estradiol and estrone inhibits sexual reproduction and skeletal growth, respectively, of corals.[12] Although we found no effects of BPA, a known estrogen receptor agonist, on asexual reproduction or development rate at the doses we tested, a higher

Table 5 AIC model selection results and parameter estimates from binomial-Poisson hurdle models that predict lacerate production of anemones treated with either solvent (2% acetone) or low or high concentrations of bisphenol A. A) Model selection results are listed in order of increasing AIC values, with the top models (ΔAIC<2) highlighted in bold. Model-averaged parameter estimates for the B) binomial hurdle and C) Poisson count estimates in the top models are listed, with non-zero estimates (based on 95% confidence intervals) indicated by a bullet (•). The binomial hurdle model results predict whether or not anemones will produce lacerates, whereas the Poisson count model results predict the number of lacerates produced among anemones that reproduced. Abbreviations: AIC = Akaike's Information Criterion, k = number of parameters, cum. = cumulative, * = both main effects and interaction were tested, : = only interaction was tested, + = only main effects were tested

A. Model selection results							B. Binomial hurdle model		C. Poisson count model	
Model	**k**	**AIC**	**ΔAIC**	**AIC weight**	**cum. AIC weight**	**Averaged Top Models Parameters**	**Estimate**	**Std. Error**	**Estimate**	**Std. Error**
null	**2**	**210.71**	**0.00**	**0.44**	**0.44**	intercept	0.75	0.69	1.10•	0.23
protein	**4**	**212.03**	**1.32**	**0.23**	**0.66**	protein	−0.30	0.23	0.07	0.08
protein+treatment	8	213.18	2.47	0.13	0.79					
protein:treatment	8	213.42	2.71	0.11	0.90					
treatment	6	214.16	3.44	0.08	0.98					
protein*treatment	12	217.04	6.33	0.02	1.00					

Table 6 AIC model selection results and parameter estimates from binomial-Poisson hurdle models that predict lacerate production of anemones treated with either solvent (2% acetone) or low or high concentrations of 17 β-estradiol (E2). A) Model selection results are listed in order of increasing AIC values, with the top model (ΔAIC<2) highlighted in bold. Parameter estimates for the B) binomial hurdle and C) Poisson count estimates in the top model are listed, with non-zero estimates (based on 95% confidence intervals) indicated by bullets (•). The binomial hurdle model results predict whether or not anemones will produce lacerates, whereas the Poisson count model results predict the number of lacerates produced among anemones that reproduced. Abbreviations: AIC = Akaike's Information Criterion, k = number of parameters, cum. = cumulative, * = both main effects and interaction were tested, : = only interaction was tested, + = only main effects were tested

A. Model selection results							B. Binomial hurdle model		C. Poisson count model	
Model	**k**	**AIC**	**ΔAIC**	**AIC weight**	**cum. AIC weight**	**Top Model Parameters**	**Estimate**	**Std. Error**	**Estimate**	**Std. Error**
protein:treatment	**8**	**224.14**	**0.00**	**0.74**	**0.74**	intercept	−0.04	0.59	0.96•	0.20
protein+treatment	8	226.70	2.57	0.21	0.95	protein:treatment solvent	−0.13	0.26	0.16•	0.08
protein*treatment	12	230.25	6.11	0.03	0.98	protein:treatment high E2	0.15	0.20	0.20•	0.05
treatment	6	232.07	7.93	0.01	0.99	protein:treatment low E2	0.18	0.27	−0.10	0.11
protein	4	234.04	9.90	0.01	1.00					
null	2	241.57	17.44	0.00	1.00					

concentration (1 mg/L) of this chemical has been shown to suppress sexual reproduction while simultaneously stimulating asexual reproduction in freshwater cnidarians.[13] The fact that estrogenic chemicals are bioactive in basal metazoans suggests that the capacity to respond to steroid hormones evolved early and that most, if not all, animals have the capacity to respond to endogenous or environmental estrogen receptor agonists. Further study of this bioactivity in cnidarians is warranted. Lastly, these data further support the use of anemones as a model organism for biomonitoring of polluted environments.[9]

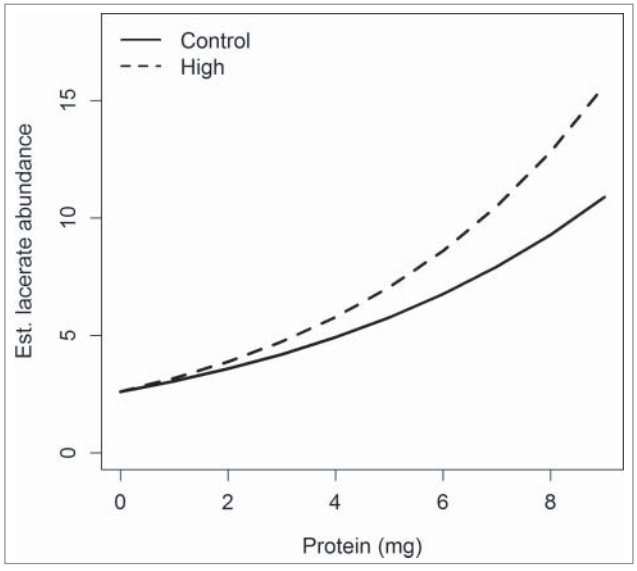

Figure 1. Effect of exposure to 17 β-estradiol on the estimated number of lacerates produced by anemones across a gradient of anemone protein content. Parent anemones were maintained individually in artificial seawater containing either control (2% acetone) or high dose 17 β-estradiol (45 ng/L) for 21 days, and the number of lacerates produced by each parent anemone was recorded. Lacerate abundance was estimated using binomial-Poisson hurdle models. Data for anemones treated with low dose 17 β-estradiol (1.8 ng/L) were excluded from this plot because parameter estimates for this group overlapped zero.

Materials and Methods

Animal husbandry and chemical treatments

Adult *A. pallida* anemones ($n = 144$) were purchased from Carolina Biological Supply Company (Burlington, NC). Anemones were maintained individually in artificial seawater (Instant Ocean, 30 parts per thousand) in glass specimen jars with glass lids. Jars were kept in a Conviron growth chamber on a 12 h:12 h light:dark photoperiod at an air temperature of 24–30°C. Anemones were fed twice weekly with *Artemia sp.* nauplii, and water was replaced 3 times weekly. Anemones were acclimated to these conditions for 15 days prior to the beginning of the experiment.

Anemones were ranked by size and then systematically assigned to one of 8 treatment groups, each containing 18 individuals, such that each treatment group contained approximately equal numbers of anemones from each size class. Treatments consisted of low and high concentrations of 17 β-estradiol (E2), bisphenol A (BPA), and bis(tributyltin)oxide (TBT), all purchased from Sigma-Aldrich®. Each chemical was dissolved in acetone and then in seawater to a final vehicle concentration of 2% acetone (**Table 1**). Low doses represent environmentally relevant concentrations,[15-17] and the concentrations of high doses were 25 times those of the low doses. Two additional treatment groups included a seawater control and a solvent (2% acetone) control.

Lacerate production and lacerate development

Lacerate production by individual anemones was monitored by inspecting the contents of each jar daily with a dissecting microscope, and the total number of lacerates formed by each anemone during the 21-day study was recorded. The approximate position of each newly formed pedal lacerate in each jar was noted, and the age at which each lacerate reached the juvenile stage (complete with stomodeum and tentacles[10]) was also recorded. Sexual reproductive performance was not evaluated in this study because the induction of spawning requires the use of a modified feeding schedule and light:dark cycle that would have confounded our analysis of asexual reproductive output.

At the end of the 21-day study, adult anemones were homogenized in artificial seawater using a glass tissue grinder with glass pestle. The homogenate was centrifuged at 8000 rpm for 4 minutes at 4°C. The concentration of solubilized protein in the supernatant of each sample was determined in triplicate by Bradford assay using bovine serum albumin (BSA) as a standard.

Statistical analyses

We used mixed binomial-Poisson hurdle models and multimodel inference[33] to test if lacerate production was predicted by protein content (as a proxy for anemone size) and/or treatment. The first set of models tested seawater versus solvent controls, and 3 subsequent sets of models included data from anemones treated with low and high concentrations of each of the 3 estrogenic chemicals along with solvent controls. In these models, the binomial distribution reflects whether or not individual anemones produced lacerates, with the hurdle function effectively constraining subsequent analysis of lacerate production to only those anemones that produced lacerates. The Poisson distribution reflects the expected structure and distribution of count data for lacerate production. We used binomial-Poisson hurdle models vs. other available zero-inflated models because we had no *a priori* reason to expect that the mechanism for inducing pedal lacerate formation was also responsible for regulating the number of lacerates produced.[34] For each combination of treatments evaluated, *a priori* model combinations of individual, additive, and interaction terms were tested, along with a null model. In addition, we used the same model combinations to test whether the age at which lacerates reached the juvenile stage was predicted by protein content and/or treatment using a generalized linear model with a Gaussian distribution.

Models predicting lacerate production and the age at which lacerates reached the juvenile stage were compared using Akaike's information criterion (AIC) model selection.[33] Models were ranked and compared by ΔAIC. We sorted competing models according to their Akaike weight and averaged the top models (ΔAIC < 2). All analyses were run using R V3.1.1,[35] and we used the pscl package[36] for hurdle models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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