Supplemental Information

Multigenerational, indirect exposure to pyrethroids demonstrates potential compensatory response and reduced toxicity at higher salinity in estuarine fish

Sara J. Hutton[1], Samreen Siddiqui[2], Emily I. Pedersen[2], Christopher Y. Markgraf[3], Amelie Segarra[4], Michelle L. Hladik[5], Richard E. Connon[4], and Susanne M. Brander[2*]

[1] Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR, 97331

[2] Department of Fisheries, Wildlife, and Conservation Sciences, Coastal Oregon Marine Experiment Station, Oregon State University, Newport, OR, 97365

[3] Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, 97331

[4] Department of Anatomy, Physiology and Cell Biology, University of California, Davis, CA 95616

[5] U.S. Geological Survey, California Water Science Center, Sacramento, CA 95819

*Corresponding author: susanne.brander@oregonstate.edu

Supplemental Information 1 - Organisms and husbandry

Inland Silversides (*Menidia beryllina*) are a model estuarine fish species approved by the United States Environmental Protection Agency for regulatory testing. They are often used in research studies as they are relatively easy to culture in the laboratory, spawn large numbers of embryos, are moderately sensitive to environmental stressors, and play a key role in estuarine food webs. Their ease of spawning allows for multigenerational studies in multifactorial experiments (DeCourten and Brander 2017). Additionally, Inland Silversides can tolerate a range of salinities, from freshwater to full strength seawater, making them an ideal species to study estuarine stressors.

Adult Inland Silverside broodstock were maintained at 10–20 PSU and 23 ºC on a 14:10 light cycle. Adult fish were fed a combination of Hikari tropical micro pellets (Kyorin Food Industries Ltd., Kasai City, Japan), Hikari freeze-dried tubifex worms (Kyorin Food Industries Ltd), Hikari frozen mysid shrimp (Kyorin Food Industries Ltd., Kasai City, Japan), and live Artemia nauplii hatched from Brine Shrimp Eggs (Brine Shrimp Direct, Ogden, Utah, USA) supplemented with Selcon™ (American Marine Inc., Ridgefield, Connecticut, USA). Brood fish were approximately 1.5-2 years old at the time of spawning. The spawning protocol was adapted from Middaugh et al. (1986) and occurred as described in Hutton et al. (2021) (Middaugh et al. 1986, Hutton et al. 2021). In order to induce spawning the salinity of the broodstock tanks was lowered using reverse osmosis water, on average the salinity changed ~3 – 5 PSU per spawn. Substrate was added to adult broodstock tanks for 16 – 20 hours following which time it was removed and placed into glass jars in a cooler to maintain the temperature of the water. Spawning substrate and embryos were subsequently transported to Oregon State University, Corvallis, Oregon, main campus, placed in 10 PSU artificial seawater (ASW) created with Instant Ocean and reverse osmosis water, and allowed to develop on the substrate until 4 days post fertilization (dpf). At 4 dpf, embryos were gently removed from the spawning substrate using forceps, placed into ASW made to their respective exposure salinities (6 and 10 PSU), rinsed, and assessed for development using a VistaVision Dissecting Scope (VWR International, Radnor, Pennsylvania, USA). They were then given 24 hours to acclimate before the exposures started.

Exposures started at 5 dpf and a 50% water change was done every 24 hours for 96 hours. Following experiment maintenance larvae were fed a combination of live rotifers, cultured in-house, and Gemma Microdiet *ad libitum* (Skretting, Westbrook, Maine). Daily water quality (pH, temperature, salinity, dissolved oxygen, and ammonia) data were collected and are shown in Table S1. Water quality data was measured using YSI Professional Plus Quatro water quality meter (YSI Incorporated, Yellow Springs, Ohio, USA) and API Ammonia, Nitrite, and Nitrate Test Kits. At the end of the 96-hr exposure period, a subset of

larvae was randomly selected for behavioral analysis (2-3 larvae per replicate, n =3/treatment). Another random subset of larvae (8-10) were selected, dried, and placed in a microtube before being frozen in liquid nitrogen and then stored at -80 C.

The remaining larvae were kept in their 1L beakers, and the exposure solution was changed three times with clean water at the appropriate salinity. Organisms were kept in the same beakers since hatching to limit handling and stress. Larvae were reared in the 1L beakers for 4-5 weeks with daily water changes and were fed artemia brine shrimp and live rotifers daily. After 4-5 weeks in 1L beakers the fish were transferred to 13L recirculating tanks where they were reared until they were sexually mature, at approximately 8 months of age. At this time the rotifers were switched out with Hikari micro pellets. Fish were fed daily, which consisted of a combination of Hikari tropical micro pellets (Kyorin Food Industries Ltd., Kasai City, Japan) and live Artemia nauplii hatched from Brine Shrimp Eggs (Brine Shrimp Direct, Ogden, Utah, USA) supplemented with Selcon™ (American Marine Inc., Ridgefield, Connecticut, USA). pH, dissolved oxygen, salinity, and temperature were recorded daily, ammonia, nitrate, and nitrite were measured weekly (Table S.2S2). At approximately 8 months post hatch Inland Silverside F0 adult fish were sexually mature and were spawned by placing substrate into the tanks for 48 hours at a time to ensure enough embryos were produced and collected for each endpoint.

To spawn the F1 generation, spawning substrate was placed in each F0 adult tank for 48 hours per spawn to ensure enough embryos were collected. Embryos were placed into 250 mL beakers with clean seawater at the correct salinity (6 or 10 PSU). Similar to the F0 larval exposures, a 50% water change was done daily, at which time debris were removed, water quality measured, and organism survival assessed. F1 water quality data can be found in Table S3. Consistent with the F0 larval exposures, F1 exposures were ended on 9 dpf (~2-3 dph).

Supplemental Information 2 - Gene Expression

RNA was extracted from 8-10 frozen Inland Silverside larvae using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacture's specifications. Total RNA concentration and 260/280 ratios (acceptable values > 2.0) were quantified using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts). RNA purity was assessed via electrophoresis on a 0.9 % agarose gel and visualized on an Azure c600 Imaging System (Azure Biosystems, Hercules, California, USA). cDNA was transcribed from RNA using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Waltham, Massachusetts, USA) according to the manufacture's specifications. Primers sequences were verified in

previous studies and were purchased from Eurofins Genomics (Louisville, Kentucky, USA). A full list of primer sequences can be found in Table S4. qPCR was performed on an Applied Biosystems QuantStudio3 in Applied Biosystems MicroAmp EnduraPlate Optical 96-Well Green Reaction Plates with Barcode (Waltham, Massachusetts, USA) with Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix.

Supplemental Information 3 - Growth and development

S3.1 F1 embryonic development

At 5 dpf the F1 embryos were assessed for development using a scoring system developed by Middaugh and Whiting, 1995 [32] and utilized in DeCourten et al. 2020 [11]. Embryos were assessed for spinal, craniofacial, and cardiovascular deformities. F0 embryos were not assessed for developmental deformities since they were already 5 dpf at the time of exposure.

S3.2 F0 and F1 larval growth index

Following behavioral assessment, F0 and F1 larvae (9 dpf/2-3 dph) were collected, euthanized with an overdose of MS222 buffer with sodium bicarbonate at 200 mg/L, placed in 3% paraformaldehyde, and stored at 4ºC until analysis. For growth index, 1 – 3 individuals from each technical replicate (n = 3) and treatment were imaged using an Olympus SZ61 Stereo Microscope and Olympus DP23 Microscope Digital Camera (Olympys Corporation, Tokyo, Japan), and length and width were measured using cellSens Imaging Software (Olympys Corporation, Tokyo, Japan). Growth index was calculated as described in Siddiqui et al. with the following equation: $^{\mathrm{W}}/_{\mathrm{L}}\,\mathrm{X\,d}$. Where *W* is width in mm, *L* is length in mm, and *d* is the number of exposure days [27].

S3.3 F0 adult development

Condition factor (GF) was measured in mature F0 adult fish as the following equation defined by Fulton: CF = $^{\mathrm{W}}/_{L^3}\,x\,100000$. Where *W* is weight in gram and *L* is length in mm. Gonadal and hepatic somatic index were measured (GSI and HSI, respectively). GSI and HIS were analyzed within sex. Sex ratio within each replicate was also calculated.

Table S.1. Water quality data for F0 Inland Silverside (*Menidia beryllina*) larvae during the 96-hour exposure period. All values reported as mean ± standard error.

	pH	Salinity (PSU)	Dissolved Oxygen (mg/L)	Temperature ºC	Ammonia (ppm)
6 PSU	8.40 ± 0.12	6.10 ± 0.07	7.29 ± 0.19	23.23 ± 0.10	0.025 ± 0.02
10 PSU	8.23 ± 0.11	10.08 ± 0.07	7.10 ± 0.09	23.04 ± 0.09	0.025 ± 0.02

Table S.2. Water quality data for F0 Inland Silverside (*Menidia beryllina*) adults during rear out period. All values reported as mean ± standard error.

	pH	Salinity (PSU)	Dissolved Oxygen (mg/L)	Temperature ºC	Ammonia (ppm)	Nitrite (ppm)	Nitrate (ppm)
6 PSU	7.84 ± 0.06	5.9 ± 0.02	7.14 ± 0.06	24.57 ± 0.03	ND	ND	34.29 ± 6.85
10 PSU	7.96 ± 0.04	9.88 ± 0.05	7.05 ± 0.06	24.28 ± 0.03	ND	ND	23.75 ± 7.30

Table S.3. Water quality data for F1 Inland Silverside (*Menidia beryllina*) embryo and larvae during the 9-day rearing period. ND = Not Detected. All values reported as mean ± standard error.

	pH	Salinity (PSU)	Dissolved Oxygen (mg/L)	Temperature ºC	Ammonia (ppm)
6 PSU	8.37 ± 0.04	6.09 ± 0.03	7.29 ± 0.07	22.74 ± 0.05	ND
10 PSU	8.54 ± 0.04	10.09 ± 0.08	7.09 ± 0.07	22.68 ± 0.05	ND

Table S.4. List of the 11 genes, functions, primer sequences, and references for sequences used for gene expression analysis.

Symbol	Gene name	Function	Primer sequence	Reference
17β-hsd	17-beta-hydroxysteroid dehydrogenase 14	Steroidogenesis	F: gatctgctcaacctcaatcttgtc R: caggtgtggcaacgcaaat	(DeCourten et al. 2020)
arx	Androgen receptor	Hormone receptor	F:atccgcatgcagtgctcata R: ccccagacctcgtattcaacg	(DeCourten et al. 2020)
cyp1a1	Cytochrome P450 1a1	Metabolism of exogenous & endogenous compounds	F: ggttgatctgccactggttg R: tggaggcttacatgctggaa	(DeCourten et al. 2020)
dntma3	DNA methyl transferase 3 alpha	DNA methylation	F:agtcgcctttaagtagtgcct R: ggggtctttggggattccat	(DeCourten et al. 2020)
esr1	Estrogen receptor alpha	Hormone receptor	F:ctccattgtgccagtgcaga R: acgcttccgcatgctca	(DeCourten et al. 2020)
igf2	Insulin-like growth factor 2	Growth factor/ development	F: gcaggtcatacccgtgatgc R: ggctgccttcctattccacac	(DeCourten et al. 2020)
atp1a1	Sodium potassium-transporting atpase alpha-1 subunit	Osmoregulation/ metabolism	F: tgatctggtggaggtgaaagg R: tgggcggagatgattctca	(Jeffries et al. 2015a)
atp1a2	Sodium potassium-transporting atpase alpha-2 like subunit	Osmoregulation/ metabolism	F: gccaacgacaacctgtacctg R: ggagaaacagccggtgatgat	(Derby et al. 2021)

rps6	40 s ribosomal protein s6	Neurodevelopment-downstream target of the mTOR pathway	F:cagcgttctcaacttg R: gaagatttgcggatccttfc	(Frank et al. 2019)
ef1a	Elongation factor 1 alpha	Reference gene	F:catcgcctgcaagttcagc R: cccagacttcagcgcctt	(DeCourten et al. 2020)
rpl_7	60s ribosomal protein 7	Reference gene	F: aacttcttgtggccgttcaag R: tcgcctccctccacaaagt	(DeCourten et al. 2020)

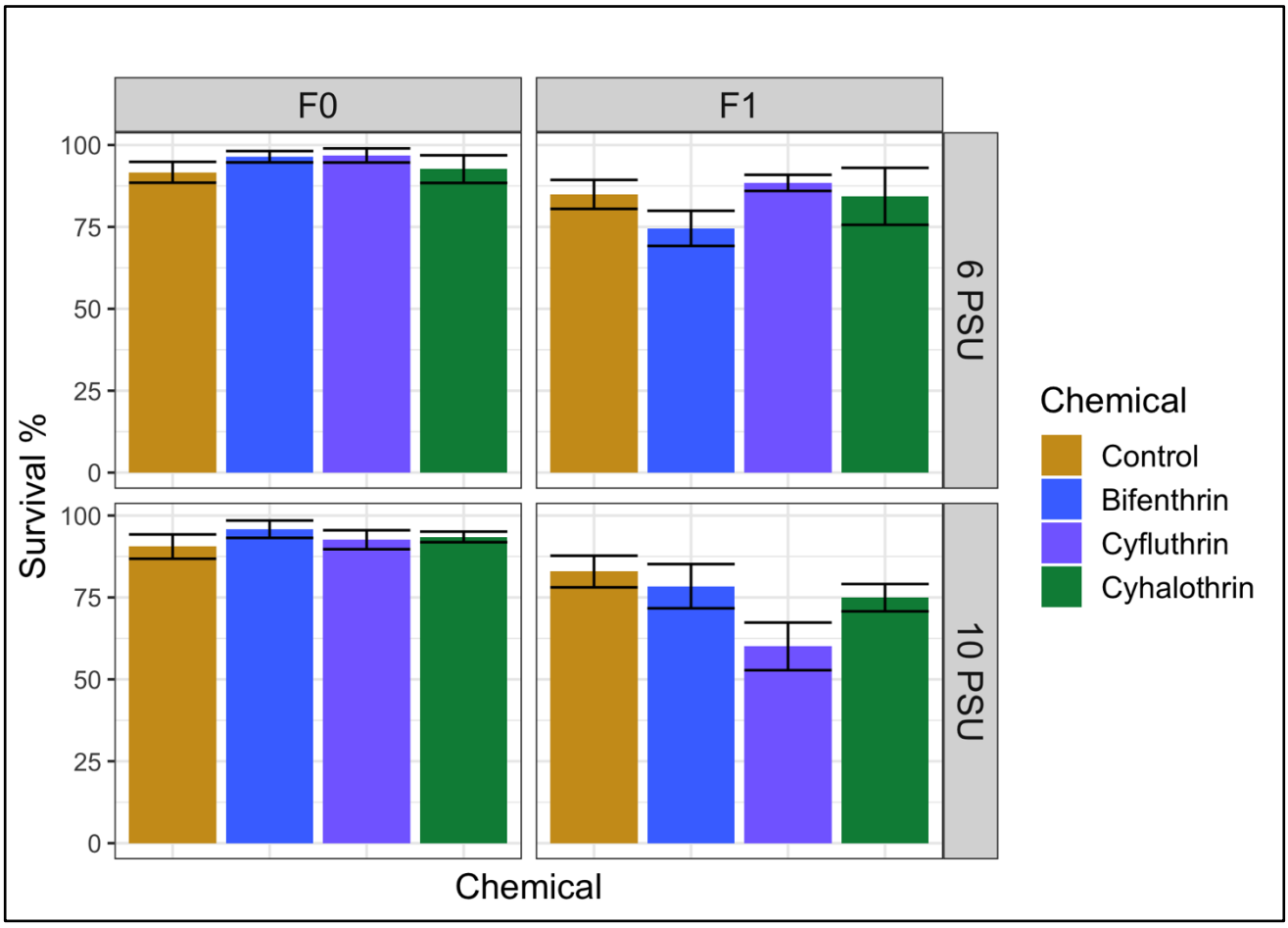

Figure S.1. Survival of each exposure scenario. Error bars represent standard error. There were no significant differences between the controls and pyrethroids within each salinity and generation exposure ($p > 0.05$, Dunnett's test). F0 larvae were directly exposed while F1 larvae where indirectly exposed to the pyrethroids through the germ line.

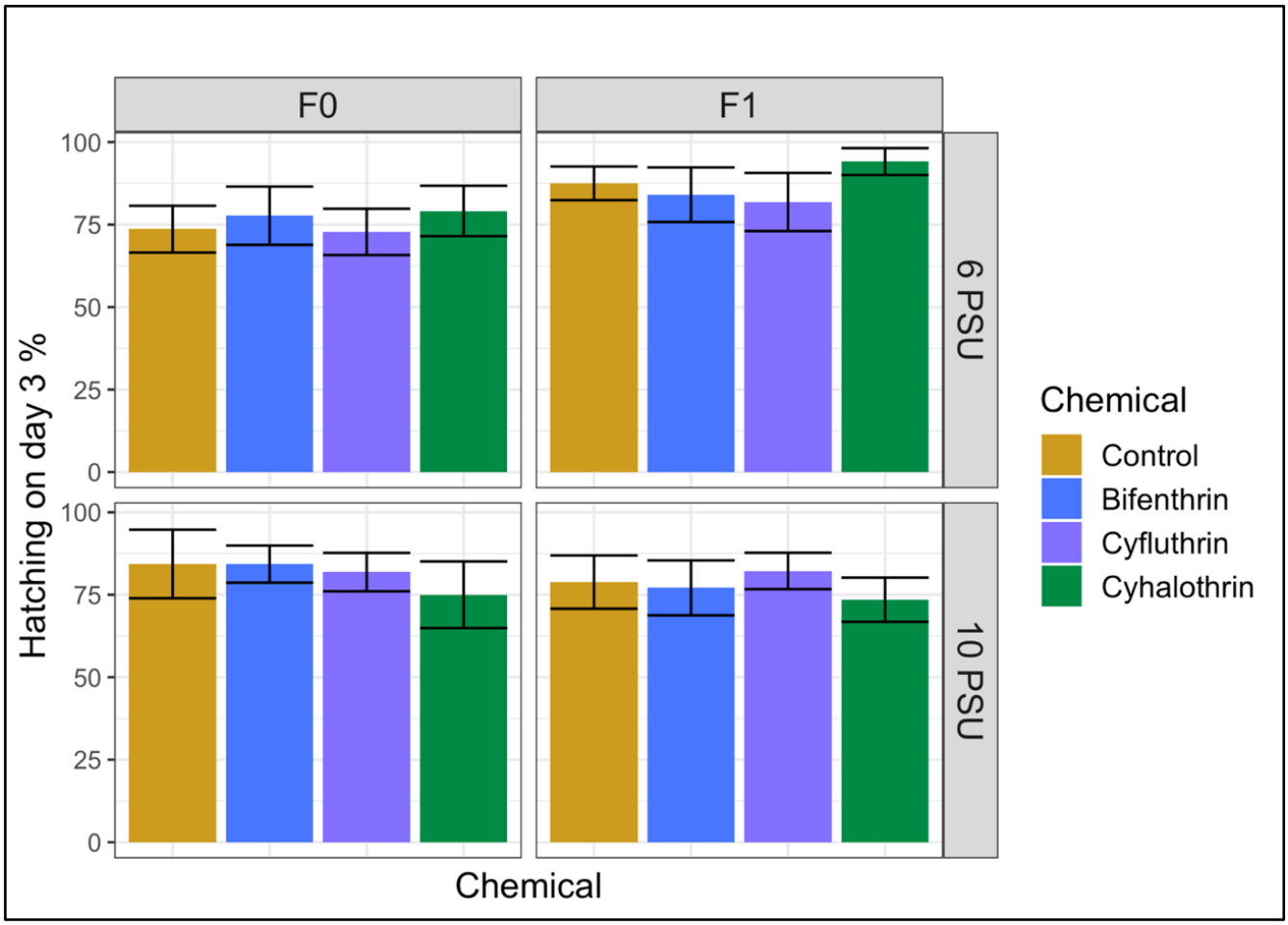

Figure S.2. Bar graphs of the percent of embryos that had hatched into larvae by day 3 of the exposure. Hatching occurred between day 1-3 of the exposure. Error bars represent standard error. There were no significant differences between the controls and pyrethroids within each salinity and generation exposure ($p > 0.05$, Dunnett's test).

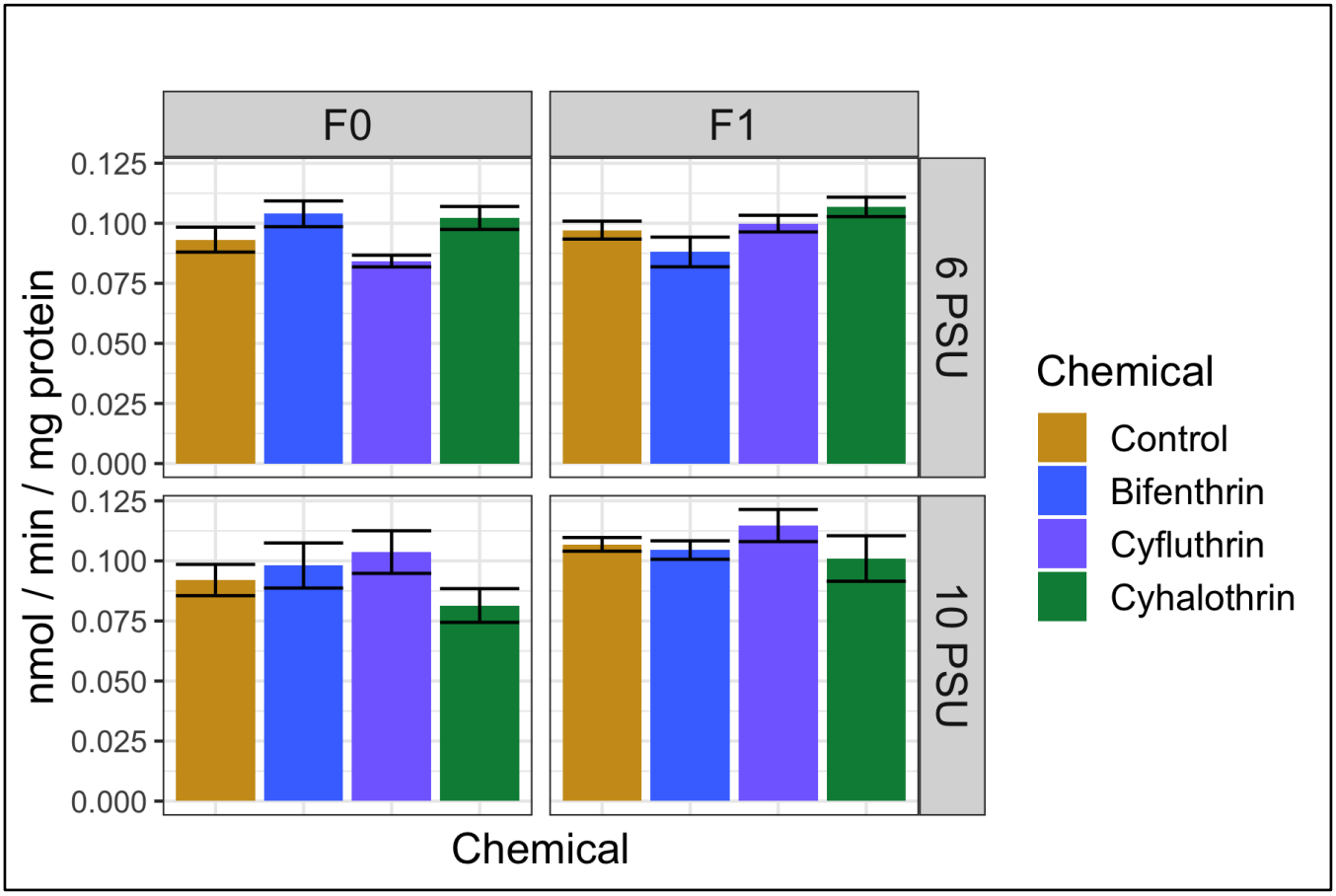

Figure S.3. Acetylcholinesterase inhibition as a measure of nmol cysteine production per minute and normalized by mg protein. Error bars represent standard error. There were no significant differences between the controls and pyrethroids within each salinity and generation exposure ($p > 0.05$, Dunnett's test).

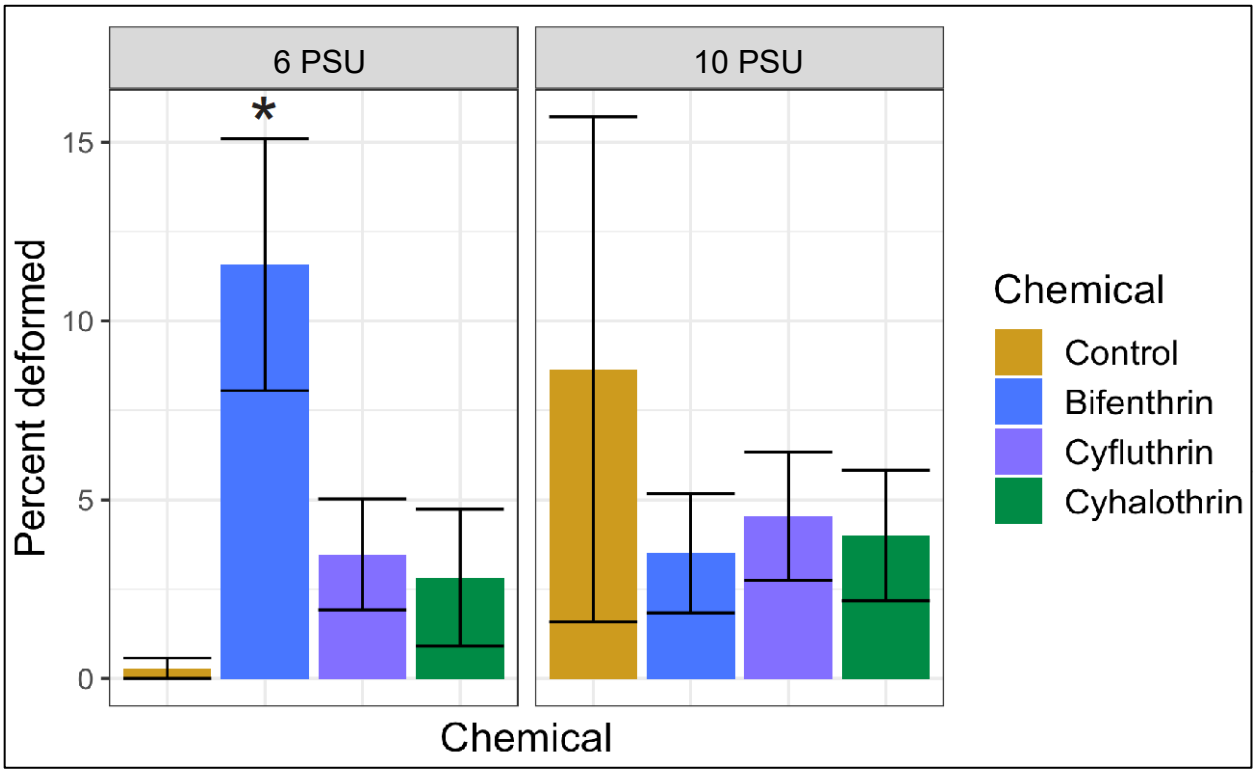

Figure S.4. Bar plot of percent developmental deformities found at 5 dpf in F1 embryos with 6 PSU on the left and 10 PSU on the right. Error bars represent standard error, and an asterisk denotes $p < 0.05$ (one-way ANOVA followed by Dunnett's Test).

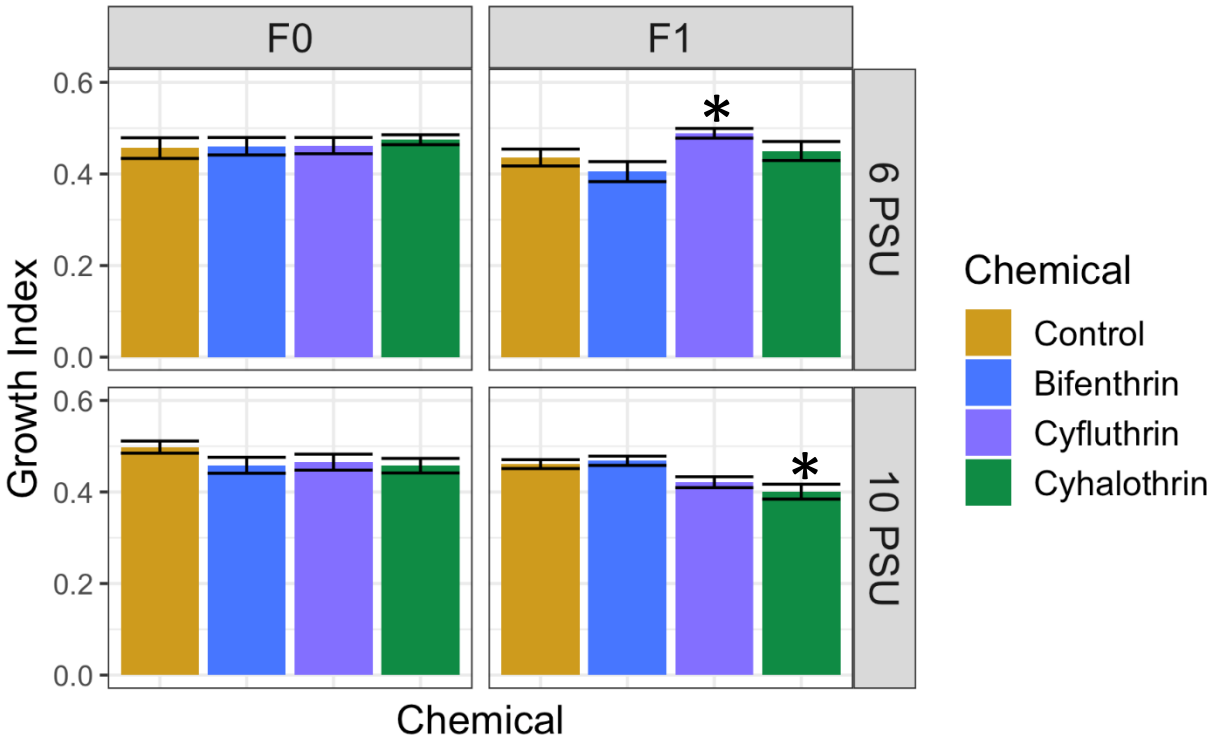

Figure S.5. Bar plot of F0 (top) directly exposed and F1 (bottom) indirectly exposed Inland Silversides larval growth index at 9 dpf. (2-3 dph) 6 PSU on left and 10 PSU on the right. Error bars represent standard error, and an asterisk denotes $p < 0.05$ (one-way ANOVA followed by Dunnett's Test).

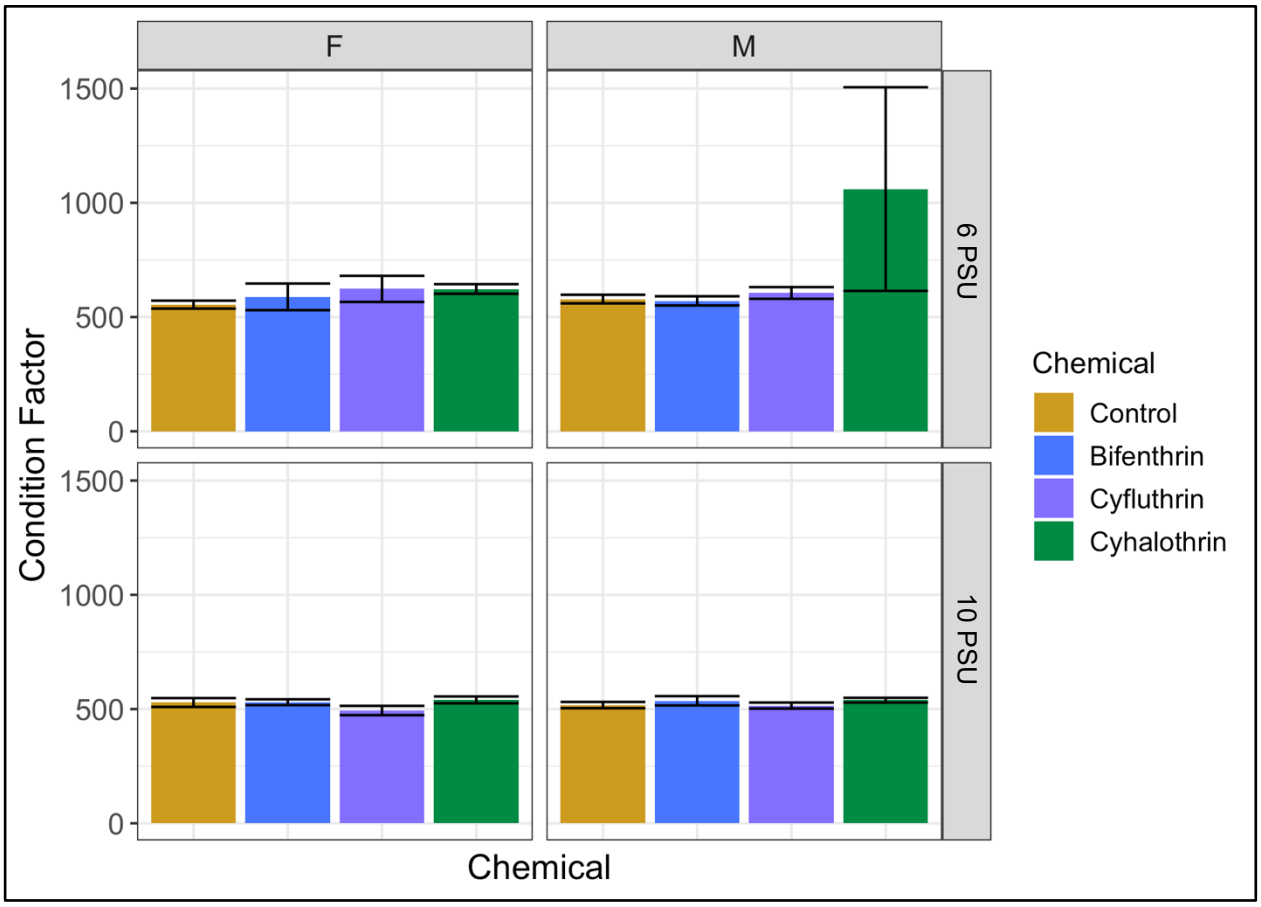

Figure S.6. Bar plot of condition factor of F0 adult Inland Silversides directly exposed to a methanol only control, bifenthrin (1.1 ng/L), cyfluthrin (0.9 ng/L), and cyhalothrin (0.7 ng/L) at 6 (top) and 10 PSU (bottom). Females (F) represented on the left and males (M) on the right. Error bars represent standard error. There were no significant differences between the pyrethroids and the control (one-way ANOVA followed by Dunnett's Test).

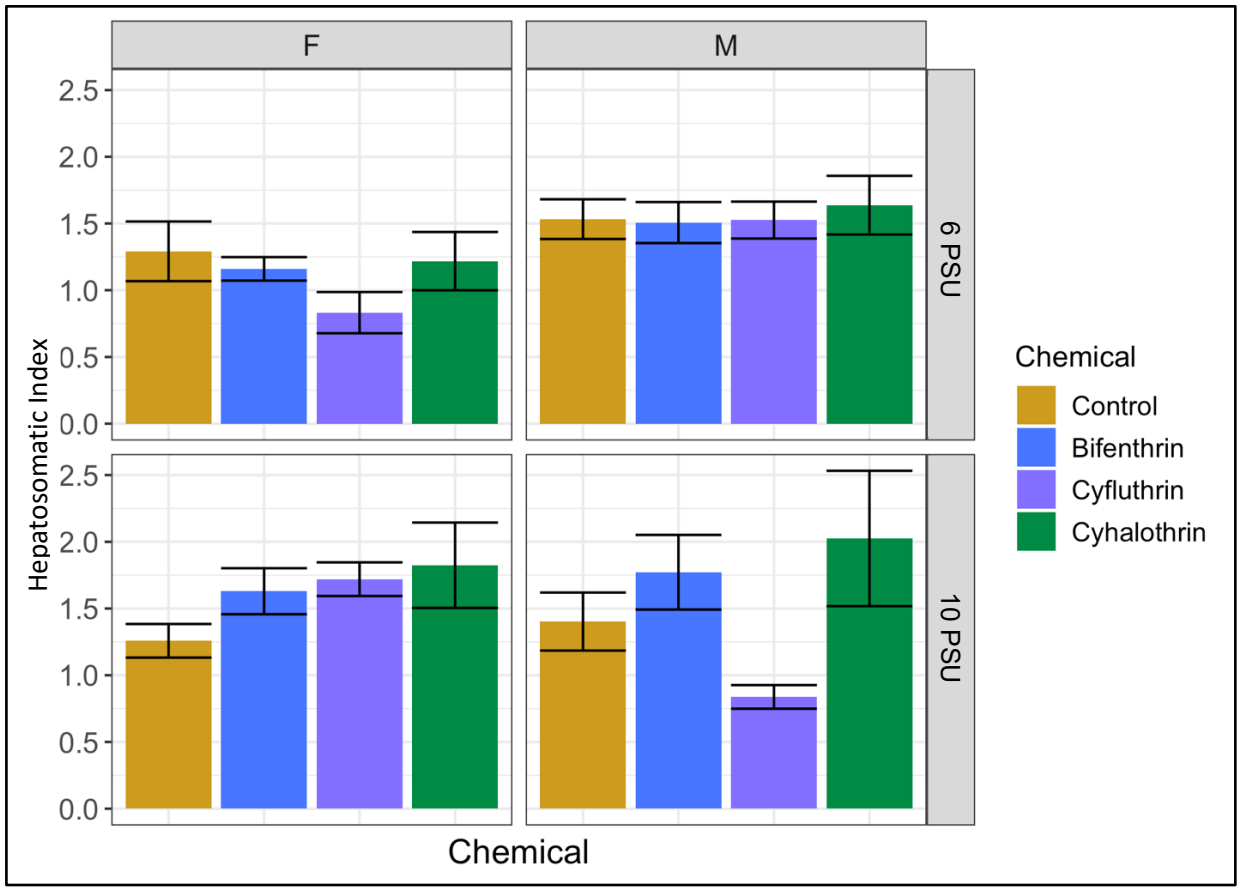

Figure S.7. Bar plot of hepatosomatic index (HSI) of F0 adult Inland Silversides directly exposed to a methanol only control, bifenthrin (1.1 ng/L), cyfluthrin (0.9 ng/L), and cyhalothrin (0.7 ng/L) at 6 (top) and 10 PSU (bottom). Females (F) represented on the left and males (M) on the right. Error bars represent standard error. There were no significant differences between the pyrethroids and the control (one-way ANOVA followed by Dunnett's Test.