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Environmentally relevant concentrations of bifenthrin affect the expression of estrogen and glucocorticoid receptors in brains of female western mosquitofish

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ABSTRACT

In recent decades, pyrethroid pesticides have been deemed a safer alternative to previously used pesticides. While some evidence supports this assumption in mammals and birds, exposure to certain pyrethroids can affect concentrations of hormones vital to reproduction in fish. Thus, we hypothesized that pyrethroid exposure impacts fish reproductive behavior and the expression of genes associated with reproduction. We tested our hypothesis by examining effects of the widely used pyrethroid pesticide, bifenthrin, on the reproductive behaviors of the broadly distributed livebearing western mosquitofish, *Gambusia affinis*. We exposed sexually mature female fish to one of five environmentally relevant concentrations of bifenthrin and conducted behavioral assays to assess reproductive, social, and space use behaviors before and after exposure. We did not detect changes in behaviors measured in response to bifenthrin. However, exposure was associated with increased expression of an estrogen receptor gene (ER-α) and glucocorticoid receptor (GR) in brain tissue at bifenthrin concentrations at concentrations of 5.90 and 24.82 ng/L, and 5.90 and 12.21 ng/L, respectively. Our study supports the perspective that the use of multiple endpoints through integrative approaches is essential for understanding the cumulative impact of pollutants. Integrating physiological, morphological, and behavioral investigations of nonlethal concentrations of pollutants like bifenthrin may heighten our potential to predict their impact on individuals, populations, and communities.

1. Introduction

Humans continue to have widespread impacts on organisms and environments globally. Many of these novel environmental impacts have appeared quickly; much faster than many species can adapt or evolve in response to them. The introduction of nonnative species, pollutants, and overharvesting have been implicated in the local extinction of many populations. While many of these human impacts may not be immediately lethal to organisms via direct effects, they may still influence the metabolism, physiology, behavior (Sih et al., 2011; Wong and Candolin, 2015), or reproductive success of individuals (or a subset of individuals) and in turn have drastic consequences for individuals or populations (e.g. Gwynne and Rentz, 1983; Kidd et al., 2007; Blazer et al., 2012; van Geffen et al., 2014; Demeyrier et al., 2016).

A wide range of toxicants in the environment have the potential to have physiological consequences for exposed organisms. Some of these compounds, such as pharmaceuticals, are inadvertently introduced through waste disposal processes. Others, such as pesticides, are intentionally released to manage particular pests. Regardless of the intended impact, these compounds were developed to influence specific biological processes in target organisms. Many of these processes are shared between target and non-target species, or have non-lethal and non-specific impacts on other biological processes in organisms. Recently, it has become apparent that more integrative approaches are necessary for assessing the impacts of toxicants on individuals, populations, and communities (Clotfelter et al., 2004; Scott and Sloman, 2004; Kumar and Holt, 2014; Saaristo et al., 2018 *accepted*). In the present study, we evaluated the impacts of exposure to a widely used

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pyrethroid pesticide, bifenthrin, in the globally introduced western mosquitofish, *Gambusia affinis*.

Bifenthrin, and other pyrethroid pesticides, have become some of the most widely used pesticides globally (Spurlock and Lee, 2008; Fong et al., 2016; Brander et al., 2016a). Brander et al. (2013) detected concentrations of bifenthrin as high as 43.0 ng/L in the San Francisco Bay estuary. A USGS report detected bifenthrin levels as high as 33.3 ng/L at sites throughout the Central Valley of California in 2015 and 2016 (De Parsia et al., 2018). The recent increase in pyrethroid use can be partially attributed to their perception as a less harmful alternative to organophosphate pesticides, which have greatly declined in use (Fong et al., 2016). Indeed, pyrethroids have been considered less harmful to mammals and birds (Spurlock and Lee, 2008); however, they have been found to have endocrine disrupting properties in fishes (Forsgren et al., 2013; Brander et al., 2016a,b). In arthropod species, which pyrethroid application targets, these compounds interfere with voltage-dependent ion channels. While they may be less harmful in some regards, pyrethroids and their metabolites also interact with steroid hormone pathways (Tyler et al., 2000; Brander et al., 2016a). Bifenthrin has been reported to be estrogenic at low concentrations, but anti-estrogenic at higher concentrations (Brander et al., 2016b), potentially as a result of negative feedback within the Hypothalamic-Pituitary-Gonadal (HPG) axis. A wide range of unintended consequences for exposed organisms have been documented including shifts in sex ratios (White et al., 2017), hormone levels and gene expression (Forsgren et al., 2013; Brander et al., 2016b; Frank et al., 2017), and developmental processes (Liu et al., 2011) including the age of onset of puberty in children (Ye et al., 2017a, b).

Poeciliid fishes are sexually dimorphic, livebearing fish that exhibit a wide range of mating behaviors, external fertilization, and maternal provisioning. Male poeciliids have evolved a modified anal fin called a gonopodium which males use to transfer sperm to females. Gonopodium development is known to be influenced by androgens and estrogens (Ogino et al., 2005; Evans et al., 2011). The gonopodium specifically, along with the diverse mating strategies exhibited by male poeciliids, have made them a useful model system for studying sexual selection and sexual conflict (Reznick, 1995; Lindholm and Breden, 2002; Langerhans, 2010; Cummings, 2015) as well as for investigating the impacts of EDCs on reproductive structures and behaviors (Howell and Denton, 1989; Angus et al., 2005; Sone et al., 2005; Toft and Guillette, 2005; Knapp et al., 2011; Tompkins et al., 2017, 2018).

The majority of studies of EDCs performed on Poeciliid fishes have been conducted using male fish (Drysdale and Bortone, 1989; Toft et al., 2003; Raut and Angus, 2010; Brockmeier et al., 2013). Many of those that did study female fish focused on the development of gonopodia-like anal fins following exposure to androgenic toxicants (e.g. Brockmeier et al., 2013). While studies focused on differences in gonopodia, or the transcriptional shifts associated with those differences, make Poeciliids a valuable model system for identifying endocrine disruption, toxicants may impact males and females through various additional biological mechanisms. Effects on females are also of particular importance to study as pollutants may not only impact the adult but also the production of eggs and their offspring (Toft et al., 2004; Ricceri et al., 2006; Rubin, 2011), having ultimate multigenerational impacts on populations (Cripe et al., 2010). Indeed, a growing body of literature indicates a need for a greater understanding of the effects of pollutants, toxicants, and pharmaceuticals on female physiology as well as female-specific reproductive processes (Braunbeck et al., 1989; Beierle et al., 1999; Hutz et al., 2006; Marentette and Balshine, 2012).

In poeciliids and other vertebrates, reproduction is largely regulated by the hypothalamic-pituitary-gonadal (HPG) axis. This system of tissues vital for reproduction is comprised of the hypothalamus (H) in the brain, the pituitary (P) that is located in close proximity to the hypothalamus near the base of the brain, and the gonads (G), namely the testes or ovaries. In poeciliids, elevated circulating 17β-estradiol concentrations in the blood are associated with the fertilization/parturition stage in females (Venkatesh et al., 1990; Ramsey et al., 2011) around the time of vitellogenesis and female peak receptivity to potential mates (Liley, 1968). While Ramsey et al. (2011) did not find an association between 17 β-estradiol and mate inspection, they reported its positive association with movement behavior. Additionally, Friesen et al. (2017) found that exposure to estradiol influenced opsin gene expression, which may have impacts on detection of and association with conspecifics, as well as selectivity for mates. In several poeciliid species, genes associated with neural plasticity can differentially express depending on whether individual females display a bias towards particular potential mates (Cummings et al., 2008; Lynch et al., 2012). For example, in guppy, *Poecilia reticulata*, exposure to 17α ethinyl estradiol (EE2) influenced neuroserpin (NS1) (Saaristo et al., 2017).

While the work of Saaristo et al. (2017) and that of others have revealed important molecular and physiological consequences of exposure to EDCs in poeciliids and other species, far fewer studies have tested the behavioral consequences of exposure, or how exposure-induced molecular and physiological changes relate to changes in behavior. Integrating these approaches can offer a greater understanding of the cumulative effects of EDCs on organisms and populations (Clotfelter et al., 2004; Scott and Sloman, 2004; Brodin et al., 2014), as well as for the establishment of tractable model systems for evaluating ecosystem health (Arcand-Hoy and Benson, 2009).

We tested the impacts of environmentally relevant concentrations of the pyrethroid pesticide bifenthrin on the behavior and gene expression of adult female western mosquitofish, *Gambusia affinis*. We hypothesized that our a priori-targeted genes under investigation, chosen because of their known roles in reproduction and survival, specifically the stress response (Table 1), would differentially express in response to exposure, and this would be associated with changes in behavior. By integrating behavioral and neuroendocrinological approaches, our results can identify potential relationships through which exposure, gene expression, and behavioral phenotypes are connected, broadening our understanding of the environmental and biological impacts of bifenthrin.

2. Methods

2.1. Gambusia affinis

The western mosquitofish, *Gambusia affinis*, is a widely distributed introduced Poecillid fish native to the central and southeastern United States. *G. affinis* and its sister species *G. holbrooki* have established populations on six continents. While the presence of *Gambusia* in their nonnative range has had consequences for native species, communities, and ecosystems, they have potential as a useful bioindicator species due to their global distribution. Mating in *Gambusia* is generally considered coercive (McPeek, 1992; Bisazza et al. 2001), but male courtship behavior (Hughes, 1985; Bisazza, 1993) and female preference for larger males (Hughes, 1985; McPeek, 1992; Lynch et al., 2012) has been reported.

2.2. Rearing, housing of fish

Fish rearing and exposure experiments were performed in accordance with the University of California, Davis Institutional Animal

Care and Use Committee (IACUC) protocol #19367. We acquired fish from Sacramento-Yolo vector control in Elk Grove, CA, and housed them in mixed sex 75 L stock aquaria for 2 months prior to experimentation in March and April 2017. During the entire habituation and experimental period, we maintained fish on a 14:10 light:dark cycle at 22 °C. We randomly placed female fish in groups of 5 and housed them in 3.75 L jars filled with 2 L of water. In total, we exposed N = 10 fish to each concentration of bifenthrin and the control, for a total of 60 fish in the study. We aerated the water in each jar, and changed 80% of the water in each jar daily (see rationale below). We fed fish *ad libitum* daily, which consisted of a mix of frozen brine shrimp (San Francisco Bay Brand, Inc.) and Daphnia (Hikari Sales USA, Inc.) (5x week), and frozen bloodworms (San Francisco Bay Brand, Inc.) (2x week). Each day, we removed leftover food from all jars during daily water changes. We housed fish in these groups of 5 for two weeks prior to the beginning of experimentation, at which time they underwent a suite of behavioral tests, then were exposed to a specific concentration of bifenthrin for 14 days. On the 15th day, we repeated the same suite of behavioral tests on each fish, and on day 16 we euthanized each fish for tissue collection between 900 and 1000 to control for circadian differences in gene expression.

2.3. Bifenthrin preparation, exposure, and validation

We purchased bifenthrin from Chem Services Inc. (purity 98.0%) and created a stock solution of 100 µg/L in a methanol solvent. Using this solution, we then produced nominal concentrations of 50 (1.18 E^{-10}), 25 (5.91 E^{-11}), 12.5 (2.96 E^{-11}), 6.25 (1.48 E^{-11}), and 3.125 (7.39 E^{-12}) ng/L (mol/L) solutions. The concentration of methanol was maintained at 0.005% in all treatments (including the control). We collected water samples from the 50, 12.5, and 3.125 ng/L treatments on one day of the experiment in 1 L amber glass bottles. These bottles were placed on ice and immediately taken to CalTest Analytical Laboratory (Napa, CA, USA) to confirm our measured concentrations (see Supplemental Table 1 for analysis results). Based on these three measured concentrations, we extrapolated missing concentrations for the other two concentrations using a linear regression. Hereafter, all concentrations listed are the measured (or extrapolated) concentrations.

Each day, we replaced 80% of the water in each jar with water containing the appropriate concentration of bifenthrin and methanol. The concentrations we utilized reflect environmentally relevant levels of bifenthrin experienced by introduced populations of *G. affinis* in the central valley of California, and previous studies in other species have identified shifts in endocrine activity after exposure to this range of bifenthrin (Beggel et al., 2011; Brander et al., 2016a, b, Frank et al., 2017).

2.4. Behavioral tests

On the day prior to bifenthrin exposure, and again on day 15 (the day after bifenthrin exposure ended), we exposed all fish to a suite of standardized behavioral tests.

We placed fish individually in a 38 L aquarium which contained two transparent plastic columns placed on either end (Fig. 1). After 10 min of habituation to the aquarium, we placed one unfamiliar male *G. affinis* in each of the two columns. These males differed in size, such that one was consistently larger than the other, as females have been shown to prefer larger males, which are purportedly of higher quality (using a protocol similar to that of Hughes, 1985; Saaristo et al., 2018. "High-quality" males averaged 26.7 mm (± 1.1 mm SD) in standard length, while "low-quality" males averaged 22.6 mm (± 0.5 mm SD). We gave fish one minute to habituate to the introduction of the males before a 10 min observation commenced. We randomized the size of the male placed on either side of the arena as well as the pairing between specific large and small males. We recorded observations using a camera mounted above the tank, and an observer naïve to the treatment of each fish scored each observation. Over the course of the observation period, we recorded the time each focal female fish spent within 5 cm (< 2 body lengths) of either male, as well as the number of visits the focal female fish made to each male.

After the mate choice test was complete, we transferred focal female fish to a divided 75 L aquaria which housed 5 unfamiliar conspecific female fish behind a transparent barrier with a removable opaque cover over it (Fig. 1). Focal fish had 10 min to habituate to the new tank before we removed the opaque barrier. After an additional 1 min, we began a 10 min behavioral observation which was also recorded from above as before. We measured the amount of time fish spent within 5 cm of the unfamiliar conspecific females, as well as the amount of the tank the fish explored during the observation (recorded as the number of squares entered and lines crossed on a 5 cm grid printed on the bottom of the aquarium). Following the second behavioral trial, we placed fish on a moistened towel resting on a scale upon which we measured and massed each fish before returning them to their home container.

2.5. RT-PCR

We removed all fish simultaneously from their home jar and euthanized them by decapitation within 30 s of the experimenter contacting the jar. We removed brains and livers and flash froze each tissue on dry ice. We froze all tissue samples within 5 min of the experimenter contacting the jar. We homogenized tissue samples and isolated RNA from the sample using the Direct-zol RNA Miniprep Kit (Zymo Research). We converted samples to cDNA using the Qiagen Quantitect Reverse Transcription Kit (Qiagen) and diluted to 50 nm/L for RT-PCR.

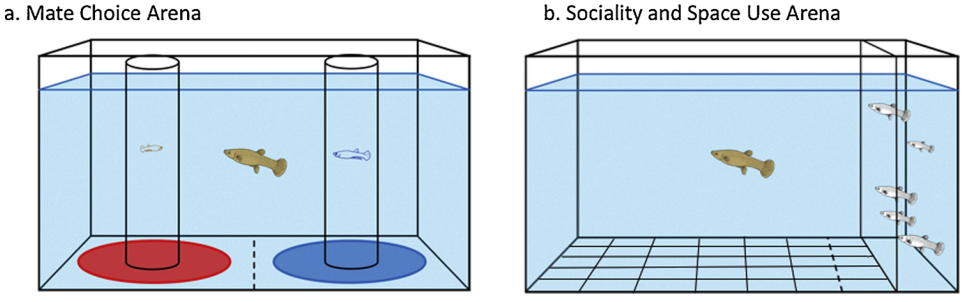

Fig. 1. Behavioral arenas for a) mate choice assessment, and b) sociality assessment. Each arena was a 37.5 L arena filled to a depth of 15 cm. In a), female fish were given a choice between associating with a smaller "low quality" male or a larger "high quality" male." Association time" was measured as the time the female spent within 5 cm (~2 body lengths) of either male. In b), "association time" was measured as the time spent within 5 cm of unfamiliar female conspecifics. In b), we also recorded lines crossed and squares entered in the arena to assess movement and exploratory behavior in the arena.

Table 1
Primers utilized for RT-PCR analysis.

Gene	Abbreviation	Tissue of Interest	Primer Sequence	RT-PCR protocol	Efficiency	Relevance
Estrogen Receptor alpha	ER-α	Brain	**F:** CTTGCCGACTCAGGAAGTGT **R:** TTTCTCTGTCACTGACGCCC	52 °C	92.1%	Associated with reproductive processes across vertebrates (Nelson and Habibi, 2013). Variation in expression associated with bifenthrin exposure (Brander et al. 2016a,Brander et al. 2016bb, Bertotto et al., 2018).
Androgen Receptor alpha	AR-α	Brain	**F:** CGTGAGAAAGCTGCACCAGT **R:** TGGAACAGGATGGGCTTGAC	52 °C	92.3%	Associated with reproductive processes and behaviors (Angus et al. 2001, Sone et al. 2005). Androgenic impacts associated with numerous EDCs (Jenkins et al. 2003, Saaristo et al. 2013)
Aromatase Cytochrome P450	Cyp19	Brain	**F:** ACAGCTACGGAAAACGTCAGT **R:** TTATGCATGAGTCCGATGTTCAG	52 °C	102.9%	Aromatizes androgens into estrogens. Known to be influenced by certain EDCs (Hallgren et al., 2006; Cheshenko et al., 2008)
Neuroserpin*	serpini1	Brain	**F**:TTATCGCTCTCTGTGGTCTGTGCT **R**:TGGGCAAATCGGATCACATAGTGG	56 °C	86.9%	Associated with female mate choice (Lynch et al., 2012; Ramsey et al., 2011; Wong et al., 2012)
Glucocorticoid Receptor	GR	Brain/Liver	**F:** GATGGAGAGCACGGCAAAAC **R:** CTGGACAGTTCTTCCTGCGG	54 °C	98.9%	Associated with stress response in vertebrates. Recently identified association with bifenthrin (Zhang et al., 2016).
Choriogenin L	ChorL	Liver	**F:** TTACGTGGACAGATGCGTGG **R:** CCTGGCATCAACCAAACACC	52 °C	103.3%	Egg precursor protein. Common indicator of estrogenic effects in fish (Arukwe and Goksøyr 2003, Inui et al., 2003)
Vitellogenin C	VtgC	Liver	**F:** TGAGCGACAACACTTCAGTGC **R:** AGCCTTTGGTCCTGGGTTATC	54 °C	97.3%	Egg precursor protein. Common indicator of estrogenic effects in fish (Arukwe and Goksøyr, 2003; Inui et al., 2003; Kidd et al., 2007)
Beta Actin (control gene 1)	ActB	Brain/Liver	**F:** CGAGACCACCTACAACAGCA **R:** CCTGGGCTGTGATCTCCTTC	54 °C	100.5%	
60S Ribosomal Protein L8 (control gene 2)	rpl8	Brain/Liver	**F:** AACTACGCCACCGTCATCTC **R:** CAGGATGGGCTTGTCGATAC	52 °C	94.6%	

*Primer designed by Dr. Mary Ramsey, see Lynch et al. (2012).

We performed a serial dilution of pooled sample specimens to determine the optimal dilution for each gene and tissue (see Table 1 for details regarding primers and specific features of the RT-PCR protocol for each gene). We performed RT-PCR using a BIO-RAD CFX384 qPCR machine. We identified and developed primers for seven genes of interest – estrogen receptor alpha (ER-α – 92.1% efficiency), androgen receptor alpha (AR-α – 92.3% efficiency), Aromatase Cytochrome P450 (Cyp19 – 102.9% efficiency), Neuroserpin (serpini1 – 86.9% efficiency), Glucocorticoid receptor (GR – 98.9% efficiency), Choriogenin L (ChorL – 103.3% efficiency), Vitellogenin C (VtgC – 97.3% efficiency). See Table 1 for details of primers and each gene of interest, and what tissue(s) their expression was measured in. We ran samples in triplicate for each of the two control genes (ß-actin, rpl8). We measured variation in expression levels once for each of the genes of interest; however ER-α and GR were measured in triplicate.

2.6. Statistical analysis

We performed a paired sample *t-test* to test whether our focal female fish had a preference for larger, "high quality" males over smaller "low quality" males, which we measured based on time spent with each male during the 10 min observation period. Because we found no preference for one male over the other (results shown below) we focused our subsequent analysis on time spent with either male. Based on our observational data, we generated several metrics to evaluate: i. changes in the time spent or visits made to either male following the exposure period, ii. changes in each fish's propensity to associate with unfamiliar female *G. affinis* following the exposure period, and iii. changes in each fish's exploratory behavior and space use (see Table 2). We analyzed these behavioral metrics using Generalized Linear Mixed Models (GzLMMs). Each of these models included bifenthrin concentration, focal fish mass, and their interaction as fixed effects. Each model also included tank identity as a random effect, as each treatment group included fish housed in one of several housing containers.

RT-PCR results for each of our genes of interest were tested for normality using a Shapiro-Wilk test. We calculated normal ΔΔCt values for each gene following methods described by Livak and Schmittgen (2001), and incorporated bifenthrin concentration as a fixed factor dependent variable and tank identity as a covariate in a Linear Model which included bifenthrin concentration as a fixed factor and tank identity as a covariate. We determined relationships between bifenthrin dose and gene expression to be statistically significant at $p < 0.05$, and marginally non-significant and worthy of post-hoc investigation at $p < 0.1$. Following these results, we performed a post-hoc Tukey's *t*-test and report significant relationships between treatment groups at $\alpha = 0.05$.

Additionally, we performed a dose response analysis on our RT-PCR results by regressing our data against various curves. Dose response analyses are increasingly recommended for toxicity studies as responses are typically not linear; thus physiologically and ecologically important relationships may be overlooked by more general approaches (Isnard et al., 2001; Cottingham et al., 2005). Following Frank et al. (2018), we tested 5 potential curves (linear, quadratic, sigmoid, and two unimodal curves) and fit each to our data and used a maximum likelihood test to evaluate whether any of these curves fit the data significantly better ($\alpha = 0.05$) than the null (intercept-only) model.

3. Results

3.1. Behavioral results

In general, bifenthrin did not cause changes in the behavioral measures we assessed. We did not identify a significant preference for large "high quality" males as opposed to smaller "low quality" males in

Table 2
Behaviors of interest.

I. MATE CHOICE AND AFFILIATION			
Behavioral Metric	*Calculation*	*Predicted Response*	*References*
Preference for "high quality" male versus "low quality" male	(sec. spent with small, "low qual." male) - (sec. spent with large, "high qual." male)	Reduced discrimination and selection for "high quality" male with increasing bifenthrin exposure. Increased avoidance of subordinate males with increased bifenthrin exposure.	Hughes (1985); Ward and Blum, 2012; Friesen et al. (2017); Saaristo et al. (2018)
Change in time spent with either male	(sec. spent with either male post-exp.) - (sec. spent with either male pre-exp)	Increased inspection of males with increasing bifenthrin exposure.	
Change in number of times either male visited	(count of visits to either male post-exp.) - (count of visits to either male pre-exp.)	Increased inspection of males with increasing bifenthrin exposure.	
II. SOCIAL BEHAVIOR			
Behavioral Metric	*Calculation*	*Predicted Response*	*References*
Change in time spent with unfamiliar conspecifics	(sec. spent with unf. consp. post-exp.) - (sec. spent with unf. consp. pre-exp.)	Increased social interactions with increasing bifenthrin exposure	Heintz et al. (2015)
Change in number of visits to unfamiliar conspecifics	(count of visits to unf. consp. post-exp.) - (count of visits to unf. consp. pre-exp)	Increased social interactions with increasing bifenthrin exposure	
III. SPACE USE			
Behavioral Metric	*Calculation*	*Predicted Response*	*References*
Change in number of squares entered during sociality assay	(count of squares entered post-exp.) - (count of number of squares entered pre-exp.)	Increased exploratory behavior with increasing bifenthrin exposure.	Dzieweczynski et al., 2014; Heintz et al. (2015)
Change in number of lines crossed during sociality assay	(count of lines crossed post-exp.) - (count of lines crossed pre-exp.)	Increased exploratory behavior with increasing bifenthrin exposure.	

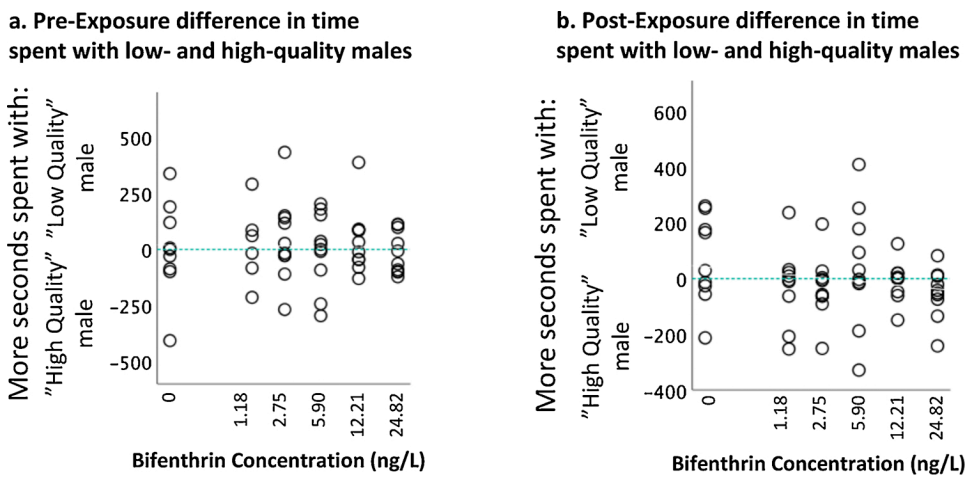

Fig. 2. Difference in the number of seconds spent with a small," low quality" male versus time spent with a large "high-quality" male before (a) and after (b) bifenthrin exposure. This difference was calculated as [seconds spent with small male – seconds spent with large male]; positive values indicate the focal fish spent more time with the small male, and negative values indicate more time was spent with the large male. There was no preference for either male in pre-exposure fish in the time spent with either male ($t_{63} = 0.602$, $p = 0.549$) or the number of visits to either male ($t_{63} = 0.256$, $p = 0.799$). Following exposure, bifenthrin exposure had no significant impact on time spent with either male (see Table # for results of GzLM). Dashed lines indicate a value of 0, or no preference.

female *G. affinis* prior to exposure to bifenthrin ($t_{63} = 0.256$, $p = 0.549$, Fig. 2a). Following the exposure period, we continued to detect no preference for either male type regardless of bifenthrin concentration ($F_{1,50} = 0.101$, $p = 0.752$, Fig. 2b). We did not find exposure to bifenthrin explained the amount of time females spent with either male ($F_{1,50} = 1.281$, $p = 0.263$, Fig. 3a), although following the exposure period females spent on average more time with males. We did not find that exposure to bifenthrin impacted the number of visits females made to either male ($F_{1,50} = 0.48$, $p = 0.492$, Fig. 3b).

We did not find a relationship between exposure to bifenthrin and the time spent ($F_{1,50} = 0.265$, $p = 0.609$, Fig. 3c) or number of visits ($F_{1,50} = 0.049$, $p = 0.787$, Fig. 3d) to a group of unfamiliar conspecific females. Additionally, we did not detect changes in the amount focal females moved through the arena (as measured by squares entered or lines crossed) regardless of whether fish had been exposed to bifenthrin (Squares Entered: $F_{1,50} = 3.357$, $p = 0.073$, Fig. 3e; Lines Crossed: $F_{1,50} < 0.001$, $p = 0.996$, Fig. 3f).

See Supplemental Table 2 for full results of each GzLM used to analyze behavioral data.

3.2. RT-PCR

We found that bifenthrin exposure explained the differential expression of two genes of interest. Our analysis indicated that exposure to bifenthrin was positively associated (although the relationship did not meet the statistical threshold for α) with changes in expression of ER-α based the results of our linear mixed models (LM: bifenthrin concentration $F_5 = 2.476$, $p = 0.056$). We performed a post-hoc Tukey's *t*-test which identified significant upregulation of ER-α at 5.90 ($p = 0.0266$) and 24.82 ng/L ($p = 0.0236$) relative to control. We found further support for this result in the results of a maximum likelihood test of best fit curves which indicated a linear curve fit the data significantly better than the null model ($p = 0.009$, Fig. 4). We found GR expression in brain tissue was significantly associated with bifenthrin concentration (LM: bifenthrin concentration $F_5 = 3.053$, $p = 0.027$). Our subsequent analysis indicated further support this relationship; we found significant differences from the control treatment between groups identified in a post-hoc Tukey's *t*-tests at 5.90 ($p = 0.008$) and 12.21 ng/L ($p = 0.0134$) for GR expression. While we found that the quadratic curve for GR expression offered the best fit of our five models, it was not a significantly better fit than the null model ($p = 0.068$, Fig. 4). Our analysis of GR expression in liver tissue did not identify variation relative to bifenthrin exposure, nor did we detect significant changes in other genes of interest in the brain or liver in any of our tests. See Supplemental Tables 3 and 4 for full results of post hoc Tukey's t-tests and LM's associated with our analysis of the relationship between exposure concentration and changes in gene expression. See Supplemental Fig. 1 for further information on changes in gene expression in liver tissues.

4. Discussion

We investigated the neuroendocrine and behavioral impacts of exposure to the pyrethroid pesticide bifenthrin in the widely introduced western mosquitofish. Following a 14-day exposure to environmentally relevant concentrations of bifenthrin, we did not observe significant changes in reproductive, social, and space use behaviors quantified. However, we did detect changes in neuroendocrine gene expression in the brain for two substrates vital for reproduction and the stress response, ER-α and GR, respectively. This may signify several non-exhaustive, and non-mutually exclusive explanations, including i) changes in gene expression may serve as a buffer against toxicant insults, preventing maladaptive changes in behavior, ii) exposure concentrations are high enough to elicit changes in gene expression, but not high enough to influence behavioral, iii) the behaviors observed are not influenced by the genes we tested, or iv) our methods of quantifying behavior were not able to detect more subtle behavioral changes which may have occurred.

A major role of estrogen receptors is to regulate the effects of estrogen (E_2) in the body, specifically those associated with reproductive processes (Muramatsu and Inoue, 2000). In female largemouth bass, *Micropterus salmoides*, from a population in Florida, USA, expression of ER-α in the liver was upregulated in February, at a time of year when estrogen (E_2) and vitellogenin levels were also elevated in female fish (Sabo-Attwood et al., 2004). That same study exposed male fish to various concentrations of E_2 and found that ER-α expression levels rose in a concentration-dependent fashion. If bifenthrin and its metabolites behave like estrogen in the body, as other studies have suggested (Brander et al., 2016a,b), then the association between bifenthrin exposure and ER-α expression we detected would be expected. It is important to highlight that while Sabo-Attwood et al. (2004) detected changes in liver ER-α, we only detected changes in brain tissue, but not liver tissue.

Estrogen receptors can also facilitate the role of estrogen neurotransmitters (McEwen et al., 2001), and can have important consequences for synapse formation (Jelks et al., 2007; Pellegrini et al., 2016). In fish, estrogens may inhibit cell proliferation and cell migration in brain tissue (Coumailleau et al., 2015). While the behavioral consequences of estrogens and estrogen receptors acting on neural development and synapse activity are not well understood, this biologically important role of estrogen and estrogen receptors may prove essential for understanding the impacts of endocrine disrupting contaminants on organisms and their behavior. Further, this is a possible mechanism through which an upregulation of genes such as ER-α

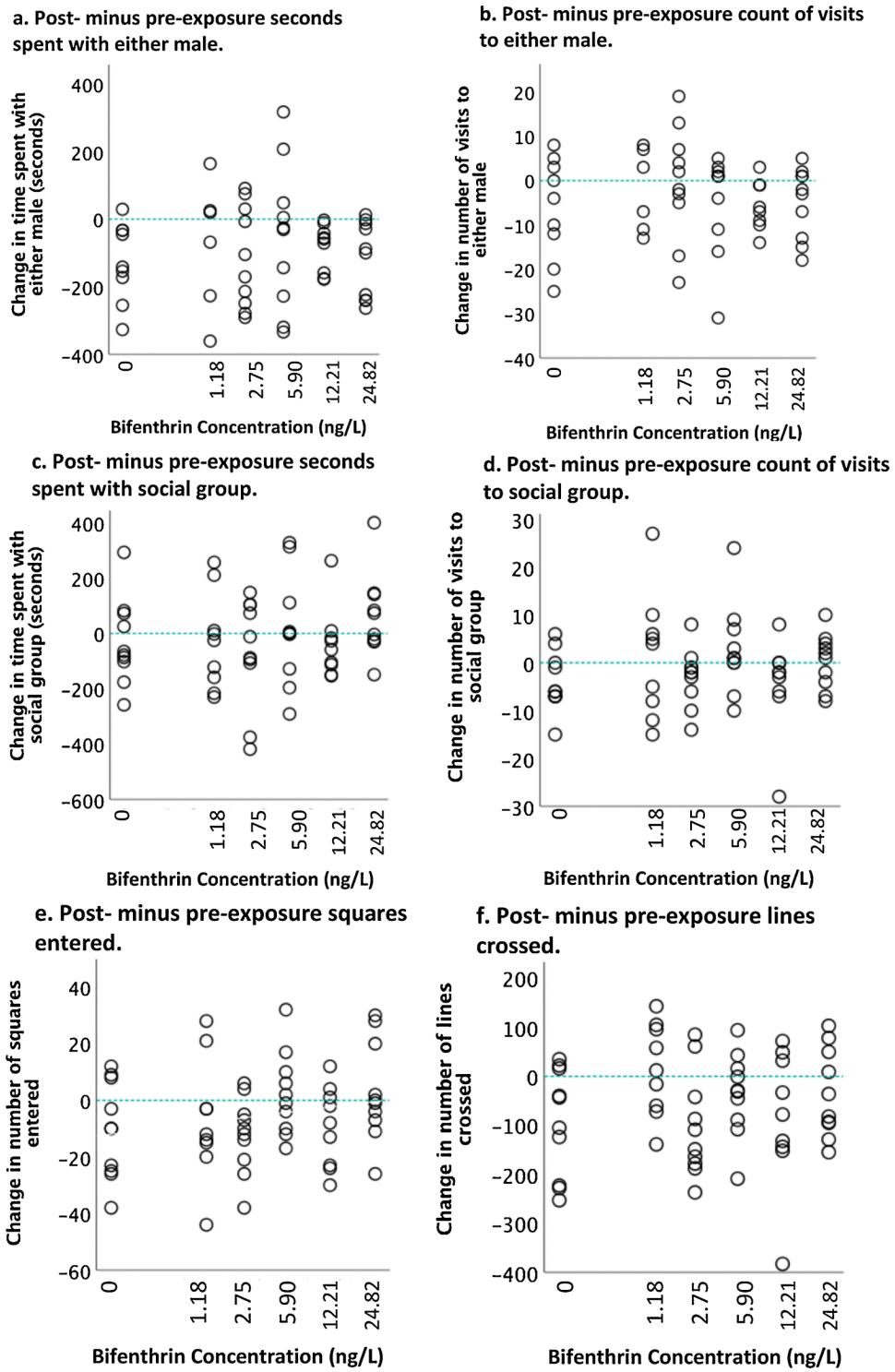

Fig. 3. Difference between post- and pre-exposure behavioral scores for a) seconds spent with any male in mate choice test, b) the number of visits to either male in mate choice test. C) and d) depict the difference in time spent with unfamiliar female conspecifics and the number of visits to conspecifics (respectively). E) depicts the difference in the number of squares entered, and f) shows the change in number of lines crossed. All metrics were calculated as [post-exp. count/time – pre-exp. count/time]. Dashed lines indicate a value of 0, or no difference.

prevents maladaptive shifts in behavior.

While we did not detect behavioral changes across treatment groups in response to bifenthrin exposure, we did identify patterns of upregulation of the expression of two genes, ER-α and GR, in the brain. This upregulation of ER-α is consistent with other studies focused on the impacts of bifenthrin on gene expression (Bertotto et al., 2018), as well as with other estrogenic compounds (Zheng et al., 2013). Zheng et al. described a biphasic response to 17β-ethinylestradiol (another estrogenic compound commonly detected in freshwater environments) exposure in another teleost fish, Crucian carp, *Carassius auratus*, such that

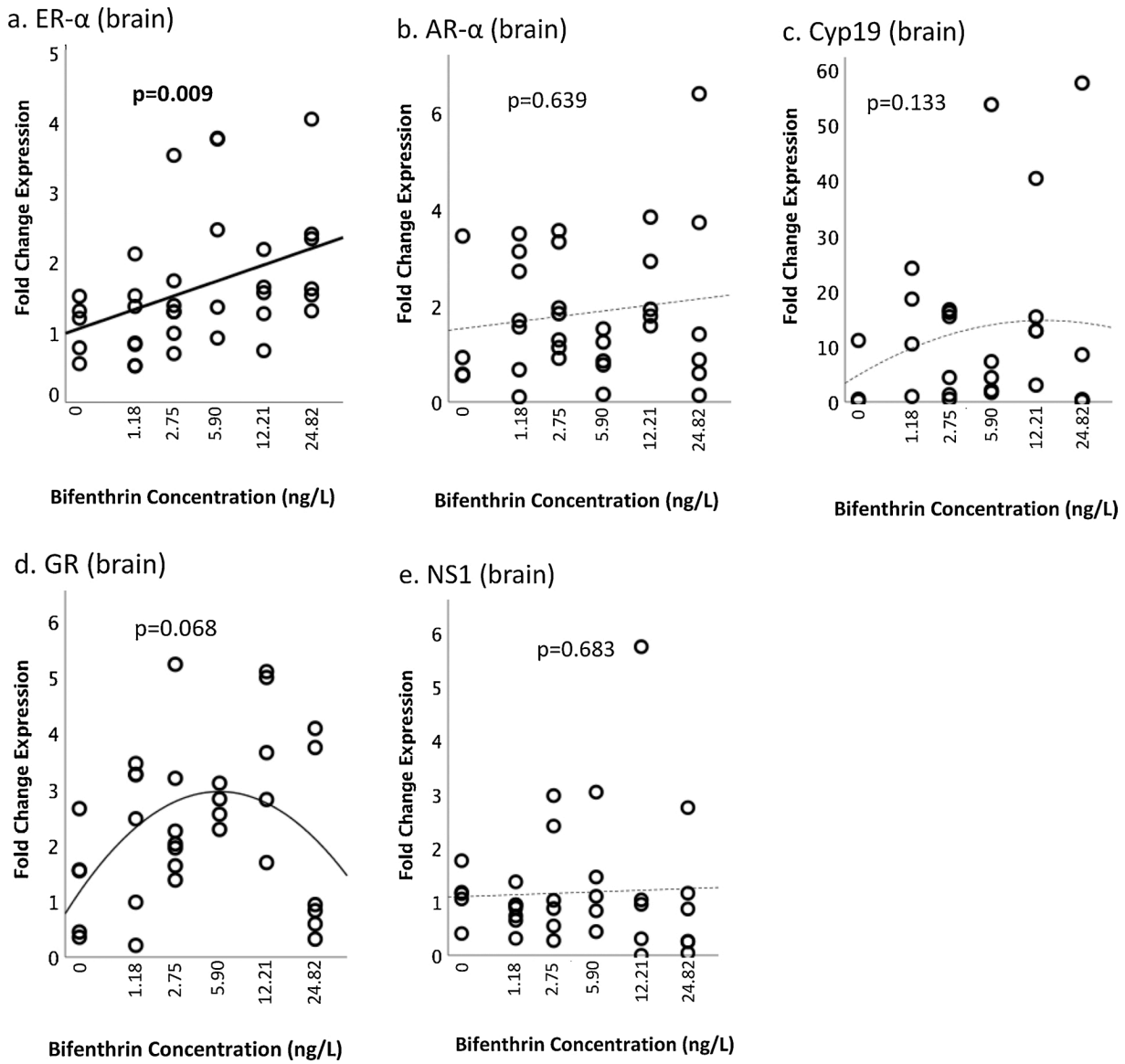

Fig. 4. Transcriptional changes in genes associated with exposure to bifenthrin in brain tissue. Transcriptional levels were measured using RT-PCR and each gene, a. *ER-a*, b. *AR-a*, c. *Cyp19*, d. *GR*, e. *NS1,* was normalized based on the geometric mean of two control genes, *ActB* and *rpl8*. Control genes were run in triplicate, as were *ER-a* and *GR*. Normalized values were evaluated using 5 possible curves (linear, quadratic, sigmoid, unimodal 1, and unimodal 2) to determine which best fit the data using a maximum likelihood approach. The best fit model is presented above, the presented p-values indicate whether each curve fits the data significantly better than the null intercept-only model. Curves with p-values in bold were significantly better than the null model ($p < 0.05$), curves with solid lines fit the data better than the null intercept-only marginally non-significantly ($p < 0.1$).

at lower concentrations, estrogen receptor (*er alpha2*) transcription was upregulated, and downregulated at higher concentrations. However, the bifenthrin concentrations of exposure we investigated were lower than those previously investigated that showed a similar pattern of response; we may have only captured the lower portion of such of a relationship for ER-α. Because our observed patterns of gene expression to physiologically relevant levels of environmental bifenthrin mirror those reported from other studies (e.g. Beggel et al., 2011; Frank et al., 2017; Bertotto et al., 2018), we suspect that the upregulation of ER-α we detected may simply be the lower half of such a biphasic response, and testing moderately higher concentrations would have been associated with a downregulation of ER-α. Interestingly, if this is the case, then the peak of the biphasic response for GR (which occurred in range of our tested concentrations) occurs at lower concentrations than that of ER-α.

Glucocorticoid receptors have recently received attention as a potential target of endocrine disrupting pollutants (Neel et al., 2013; Zhang et al., 2016). Glucocorticoids perform a wide range of functions in the body, but are generally associated with the stress response as they are released in response to stressors (Sapolsky et al., 2000; Romero and Wingfield, 2015). Sathiyaa and Vijayan (2003) exposed rainbow trout, *Oncorhynchus mykiss*, to cortisol and found an upregulation of GR mRNA. We did detect a clear nonmonotonic response in expression of the GR receptor in brain tissue. To our knowledge, ours is the first study

to detect an association between bifenthrin and GR gene expression in vivo. Zhang et al. (2016) identified bifenthrin as a GR antagonist in vitro, and this effect is consistent with our observed response.

In contrast to previous studies on the effects of bifenthrin, we did not find evidence of estrogenic effects in liver tissue. Neither ChorL or VtgC, egg proteins induced by estrogens, differentially expressed in response to treatment concentrations of bifenthrin exposure. Beggel et al. (2011) and Brander et al. (2012) had reported effects in vitellogenin and choriogenin gene expression (respectively), but their subjects were larval or juvenile fish. We tested the effects of bifenthrin exposure on reproductively active adult females, whose ChorL and VtgC transcription capabilities may already be organized during development and upregulated for reproduction.

Various pollutants have been reported to impact sociality and general activity, behaviors that can influence an organism's ability to avoid predation (Scott and Sloman, 2004). For example, in many fish species, exposure to pollutants explains changes in levels of activity (Ramsey et al., 2011; Marentette and Balshine, 2012; Renick et al., 2016, Frank et al. 2018), social interactions (Toft et al., 2004; Dzieweczynski et al., 2014, 2018; Heintz et al., 2015), and response to predation cues (Sih et al., 2004; Marentette and Balshine, 2012). However, we did not find evidence of bifenthrin exposure impacting sociality or movement in reproductively mature female *G. affinis*. Thus, while our experimental manipulations of bifenthrin exposure elicited changes in gene expression, these changes in gene expression may not have been great enough to elicit significant behavioral responses that could be captured by our measurements.

Another potential reason for the lack of observed behavioral changes in response to bifenthrin exposure is that females may have not been receptive to, or have a preference for, potential mates. Reproduction in *G. affinis* is seasonal, and is typically reduced in winter when water temperatures are lower and daylight is shorter (Edwards et al., 2010). However, during this same period, non-experimental *G. affinis* housed in stock tanks with identical conditions were successfully mating and reproducing, offering confidence that our housing conditions were indeed suitable for mosquitofish reproduction. Additionally, we isolated females prior to experimentation, which in previous studies has been important for eliciting female interest in potential mates (Hughes, 1985; Lynch et al., 2012). Alternatively, females in our study may have been receptive to potential mates, but lacked a strong preference for large males in the absence of other males attempting to sneak copulations (which could be prevented by associating with a large, guarding male - Lynch et al., 2012). Previous studies that detected preference for male size in *G. affinis* also tested other Poeciliid species with clearly defined male courtship behaviors (e.g. Lynch et al., 2012) and found far greater female preference in these courting species. Other studies of *Gambusia* species have found no female preference for males of a certain size class (Bisazza and Marin, 1991). In Poeciliid fishes in which all males primarily use sneaking behaviors such as *G. affinis*, female choice also may be more clearly evaluated in the context of mate avoidance (Magurran and Seghers, 1994) or repelling particular males (McPeek, 1992), neither of which were evaluated in our study.

The Central Valley of California has a long history of pesticide use and pollution, and while we don't know the specific history of our fish, it is possible that our source population has had time to adapt to pesticides such as bifenthrin. Recent work has shown evolutionary change in response to human impacts on environments. Reid et al. (2016) found that populations of Atlantic killifish (*Fundulus heteroclitus*) in urban estuaries had rapidly adapted to high concentrations of toxicants, and that this tolerance was associated with selection for toxicity-mediating genes. Similarly, Jayasundara et al. (2017) also found evidence of selection favoring certain genotypes killifish living in polluted environments. Their study indicated that selection for these genes may come at a cost in terms of physiological function and constrain habitat selection.

In summary, we identified changes in neural transcription, but not behavior, in adult female *G. affinis* in response to environmentally relevant concentrations of the widely used pesticide, bifenthrin. Our findings demonstrate that a lack of a behavioral effect in the presence of a toxicant does not necessarily imply the lack of a neural effect, other potential impacts to biological systems and future reproductive success resulting from changes in gene expression warrant further study. Using integrative approaches to investigate the impacts of pollutants on organisms may shed light on such repercussions, broadening our scope of the consequences of human-made toxicants on the survival and reproduction of organisms.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2018.12.001.

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