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Bifenthrin exposure causes hyperactivity in early larval stages of an endangered fish species at concentrations that occur during their hatching season

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Abstract

Bifenthrin is a pyrethroid insecticide commonly used in agricultural and urban sectors, and is found in watersheds worldwide. As a sodium channel blocker, at sublethal concentrations it causes off-target effects, including disruption of calcium signaling and neuronal growth. At the whole organism level, sublethal concentrations of bifenthrin cause behavioral effects in fish species, raising concerns about the neurotoxic properties of the compound on fish populations. Here we describe the application of a high-throughput behavioral system to evaluate contaminant impacts on the sensitive early-life stages of Delta smelt (*Hypomesus transpacificus*), a critically endangered teleost species endemic to the San Francisco Bay Delta (SFBD), California, USA.

Leveraging the natural behavior of early-larval Delta smelt, whereby they increase movement in bright light and decrease movement in the dark, we developed a test using a cycle of light and dark periods in a closed chamber to test hyper- or hypoactivity for this species. We show that early-larval Delta smelt have a significant preference to move toward light, and utilized the behavioral

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test to evaluate the impact of exposure to bifenthrin at concentrations found in habitats where Delta smelt reportedly spawn, ranging up to concentrations detected in tributaries to these habitats. All tested concentrations of bifenthrin (nominal 2, 10, or 100 ng/L) caused hyperactivity, over a 96 h exposure, with noted significance determined during the light period of the test. To further understand the impact of bifenthrin exposure, expression of a suite of genes relevant to neurodevelopment, the mechanistic target of rapamycin (mTOR) signaling pathway, and biotransformation in exposed larvae were also measured. Following exposure to picomolar concentrations of bifenthrin, expression of genes in the mTOR signaling and neurogenesis pathways were altered alongside behavior. This study demonstrates how light and dark cycle behavioral tests can be used to assess sensitive alterations in swimming activity in Delta smelt at early developmental stages and how gene expression can complement these assays. This approach can be used to assess the impact of multiple compounds that occur within the restricted habitat of Delta smelt, thus having the potential to greatly inform conservation management strategies for this critically sensitive life stage.

Keywords

Delta smelt; *Hypomesus transpacificus*; fish behavior; pyrethroid; Ca^{+}-dependent signaling; conservation

1. INTRODUCTION

Pyrethroids have grown in popularity as insecticide treatments in agricultural and urban sectors since the 1970's, across the world and in the United States (Kuivila et al., 2012). Bifenthrin is among the most commonly detected pyrethroids in irrigation, storm water run-off, and sediments (Tang et al., 2018). Because bifenthrin is used heavily in urban applications, it often enters watersheds, particularly after storm events (Weston et al., 2019) following which concentrations up to 106 ng/L have been reported in Californian surface waters (Weston and Lydy, 2012). Because of its prevalence, the impacts of bifenthrin on both vertebrate and invertebrate aquatic organisms are of concern. Further, because concentrations found in the watershed are typically sublethal to fish, assays that use endpoints other than mortality are of importance to interpret environmentally-relevant effects of these compounds and possible interpolation of results to population level impacts (White et al., 2017).

Pyrethroids are classified as Type I or Type II according to their chemical structure. Bifenthrin is considered a Type I pyrethroid insecticide, as it lacks an α-cyano group on the phenoxybenzyl moiety, but has an effect characteristically similar to Type II pyrethroids (Gammon et al., 2019); potentially causing whole body tremors as well as choreoathetosis (involuntary contractions and twisting; Yang and Li, 2015). The molecular target of bifenthrin is the insect voltage gated sodium channels (VGSCs), but it also has the capability of binding vertebrate sodium channels (Soderlund, 2012). Binding to VGSCs, delaying closure of the channel, results in prolonged depolarization of neurons, ultimately resulting in convulsions and death. At sublethal concentrations, bifenthrin has been found to induce hyperactivity in fish and rats (Frank et al., 2019, 2018; Richardson et al., 2015).

Bifenthrin has been found to alter calcium oscillations in cultured rodent neurons, independent of sodium channel activity, as well as increase neurite outgrowth at nanomolar levels (Cao et al., 2014). Calcium plays many diverse roles in neurodevelopment and can elicit alterations in neurobehavior when disrupted (Gargus, 2009). *In vivo*, larval zebrafish (*Danio rerio*) exposed to bifenthrin experienced delayed hyperactivity in behavioral tests, and altered transcription of genes dependent on Ca^{2+} signaling (Frank et al., 2018). Known to be activated by intracellular calcium release, the mechanistic target of rapamycin (mTOR) signaling pathway plays a significant role in neurodevelopment, neuronal function, and skeletal muscle function and growth (Takei and Nawa, 2014). Biotransformation, the process by which natural or environmental substances are chemically altered within an organism, can also affect development. Changes in expression of transporters and enzymes involved in this process can indicate that the organism may be expending additional energy on these protective strategies, possibly resulting in developmental delay or alteration (Hamdoun and Epel, 2007). Collectively, these findings illustrate the potential of bifenthrin to impact the neurodevelopment process.

Fish species are especially vulnerable to toxicants and other abiotic insults during their early life stages (Hamdoun and Epel, 2007), and may be particularly at risk of adverse effects from bifenthrin exposure. The Delta smelt (*Hypomesus transpacificus*) is a euryhaline teleost species that is endemic to the San Francisco Bay Delta (SFBD). Now listed as a critically endangered species, Delta smelt abundance has rapidly declined since the mid-1980's (Hobbs et al., 2017). The reason for decline in population is most likely the product of numerous elements, including the species' increased sensitivity to environmental change and anthropogenic stressors. Physical habitat parameters, especially temperature, salinity, and turbidity, correlate strongly to Delta smelt distribution in the SFBD (Brown et al., 2016).

Often acclaimed as an indicator of environmental health of the SFBD, precipitous drops in Delta smelt abundance suggests that the ecosystem is undergoing large shifts in function (Lessard et al., 2018). Although this species has substantial impacts in management of the SFBD, remarkably little is known regarding its early life stages. Observations of early larval development in the wild are scarce, creating a gap in knowledge of this critical life stage. Laboratory studies have been conducted to explore early life stages and required optimal conditions (Romney et al., 2019), adding to the strength of the base of knowledge needed to conduct toxicological experiments in the laboratory with this species. Considering the eventual goal of re-introduction of the captive refuge culture into the wild (Hobbs et al., 2017; Lessard et al., 2018), the development of toxicology tests, which can be conducted under controlled laboratory conditions, will serve to gather information on best practices for release. Because the entire distribution of the endangered Delta smelt is confined to the SFBD, understanding interactions with contaminants prominent in the area is of heightened importance, as exposure is likely to lead to greater habitat compression.

Behavioral tests can be powerful tools to assess the sublethal effects of chemical compounds. To this effect, we have designed a photomotor behavioral assay after tests previously designed for model species such as zebrafish and fathead minnow (*Pimephales promelas*) used to assess a wide array of neurological phenotypes (Dach et al., 2019; Steele

et al., 2018). Particularly, photomotor behavior in response to oscillating light and dark periods has been used in zebrafish as a behavioral endpoint in larvae (Dach et al., 2019). The behavior observed by zebrafish (lack of movement, or 'freezing' in the light, and increased movement in the dark) has been described as a passive fear response to the light stimuli (Rennekamp et al., 2016). Fathead minnow exhibit a similar yet opposite behavior pattern, moving in the light and decreasing movement in the dark (Steele et al., 2018). Zebrafish and fathead minnow behavior can be disrupted by the addition of neuroactive compounds (Steele et al., 2018). Taking this varying response to light into account, we investigated the photomotor response of Delta smelt larvae in a similar light and dark cycle test, referred to here as the Light-Dark (LD) Cycle assay. Bifenthrin is often among the most commonly detected pyrethroids throughout SFBD (Deng, 2017). It has been measured at concentrations in ng/L range, up to 32.2 ng/L in creeks and rivers in the SFBD, as well as up to 106 ng/L in agricultural and urban drainage areas adjacent to habitats in which Delta smelt have been sampled (Deng, 2017; Weston et al., 2019). Based on the current literature involving other teleost species, we hypothesized that exposure of Delta smelt larvae to environmentally relevant concentration of bifenthrin would result in hyperactivity, and that the expression of genes related to neurodevelopment would differ when exposed.

The primary purpose of this investigation was to determine if environmentally-relevant bifenthrin concentrations impact or alter the behavior of early stage larval Delta smelt, and whether changes in expression of genes relevant to neurogenesis, the mTOR signaling pathway, and biotransformation could help understand the mechanisms underlying impacts of exposure on larval development. To this effect, we adapted a behavioral assay that has been validated for use on other teleost larvae species, for use with larval Delta smelt. The selection of genes of interest was driven by responses identified in similar prior studies on zebrafish (Frank et al., 2018) and Inland silversides (*Menidia beryllina*; Frank et al., 2019).

2. METHODS

2.1. Fish source

Delta smelt embryos were fertilized via strip spawning (two females and two males) at the UC Davis Fish Conservation and Culture Laboratory (FCCL), Byron, CA, approved by the University of California Institutional Animal Care and Use Committee (IACUC) protocol #19841. Embryos were maintained at the FCCL until 7 days post fertilization (dpf) (Lindberg et al., 2013). At 7 dpf, embryos were transported to the UC Davis School of Veterinary Medicine (SVM) and held at 16°C for all tests. At this temperature, Delta smelt larvae are known to hatch between 8 and 10 dpf (Romney et al., 2019) and initiate exogenous feeding within 5 days post hatch (dph), before their yolk-sac is depleted (Baskerville-Bridges et al., 2005). All tests were conducted on 10, 11, or 12 dpf larvae, ending all tests prior to feeding requirements. These developmental time points are also advantageous such that the same response can be measured over a standardized 96 h exposure period (EPA, 2002)

Research conducted on early larval stages was approved by IACUC protocol #20705 For all experiments described, except the pesticide exposure, embryos were randomly distributed into glass petri dishes with 100 mL filtered ground water (0.2 μm), sourced from the UC

Davis Center for Aquatic Biology and Aquaculture, Davis, CA, with 200 embryos per dish, as described in the methods of Romney et al. (2019).

For all the tests, water quality was measured on day 1 (8 dpf), and the last day of the test (12 dpf, with daily 50% water replacement from an aerated carboy). Temperature ranged from 16 – 16.4°C (Hanna Instruments, Woonsocket, RI, USA), dissolved oxygen ranged from 9.82 – 10.43 mg/L (YSI, Yellow Springs, OH, USA), pH 8.55 – 8.61 (Hanna Instruments), salinity was 0.4 PSU (Hanna Instruments), and ammonia was 0 (API, McLean, VA, USA).

2.2 LD cycle assay development

We developed a behavioral test that leveraged the observed natural photomotor response of the larvae, referred to here as the Light-Dark (LD) cycle assay. In preliminary trials of the LD cycle assay, larvae of 10, 11, and 12 dpf responded in a more robust manner (as compared to 8, 9, or 13 dpf larvae). At the age of interest (10 dpf, 11 dpf, or 12 dpf), larvae were carefully transferred using a 1 mL plastic pipette into a (non-treated) 12-well cell culture plate (Thermofisher #150200, San Diego, CA, USA) containing 2mL of filtered water, with one larva per well. The larvae were allowed to acclimate to the plate conditions for at least 1 h before transferring the plate into a DanioVision Observation Chamber (Leesburg, VA, USA), equipped with infrared and visible light sources. Once placed in the chamber, larvae were allowed to acclimate in the dark for at least 5 minutes. Larvae were filmed from above, illuminated with programmable light from beneath the plate, set at 10,000 lux for each light cycle. Tracking began with 10 min dark period (Dark 1), followed by 5 min light (Light 1), 10 min dark (Dark 2), 5 min light (Light 2), and a final 10 min dark period (Dark 3). The temperature of the plate was kept at 16 °C throughout the duration of the test via a recirculating water system attached to chiller (TECO-US, Terrell, TX, USA).

2.3 Light period housing effects on movement in the LD cycle assay

In order to assess the effects of different housing conditions on larvae performance in the LD cycle assay, larvae were kept in either 24 h dark conditions, or a 12:12 h light:dark condition, from the day of transfer from FCCL (7 dpf), both at 16 °C. At the ages of interest (10 dpf, 11 dpf, or 12 dpf), larvae were assessed in the LD cycle assay as previously described (in section 2.2), in which plates were randomly distributed with 6 larvae from each condition (n=2 plates, biological replicates n=11–12).

2.4 Light-Dark preference test

In order to investigate the extent to which larvae were moving towards or away from the light, a preference test was conducted. A custom-made light-dark, IR light translucent grid (Ethovision), which allows the tracking of the larvae to continue in the dark periods, was used to test preference. This grid was placed underneath the 12-well plate, creating a “covered” area and a “light” area in each well. The behavior of larvae at 10, 11, or 12 dpf was assessed using the gridded system in the LD Cycle assay (n=2 plates, biological replicates n=23–24). The preference test was also performed with an extended light and dark period, which included a 5-minute acclimation to dark, tracking of 20 minute of Light (Light), followed by 20 minutes of dark (Dark) (n=3 plates, biological replicates n=19–36).

Total time spent in either the open or covered well was measured in the LD cycle assay as well as the extended 20 min preference assay. The proportion of time spent in each area was calculated by dividing seconds recorded of larvae in said area by total seconds recorded of larvae in arena (binned per minute and averaged over cycle).

2.5 Bifenthrin exposure

2.5.1 Chemical information—Larvae were exposed to nominal concentrations of 2 ng/L [4.73 picomolar], 10 ng/L [23.65 picomolar], or 100 ng/L [236.5 picomolar] of bifenthrin (ChemService, WestChester, PA, USA. CAS: 82657-04-3, product #: N-11203-100MG). Concentration range was chosen to reflect environmentally relevant concentrations typically found in the SFBD and its tributaries (California, USA) (Deng, 2017; Weston et al., 2019). Methanol, not exceeding 0.02% v/v, was used as a vehicle solvent carrier for bifenthrin. The bifenthrin stock solution concentration was verified prior to exposure, by evaluating aqueous concentrations representing nominal test concentrations of 10 ng/L and 100 ng/L; these were determined by CalTest Analytical Services (Napa, CA, USA), to be 5.9 ng/L [13.95 picomolar] and 76 ng/L [179.7 picomolar], respectively. No bifenthrin was detected in control samples. The analytical methods were conducted according to the United States Environmental Protection Agency (EPA) method EPA 8270M NCI (EPA, 2018), with a reporting limit of 0.5 ng/L and a method detection limit of 0.1 ng/L.

2.5.2. Bifenthrin exposure parameters—On receipt, embryos were randomly distributed into 200 mL glass beakers filled with 100 mL filtered (0.2 μm) ground water with a stocking density of 20 larvae per beaker. Each treatment (concentration and time) was repeated six times with each replicate of each treatment as an exposure beaker (n=6 beakers per treatment). There were four exposure treatments: vehicle control and three bifenthrin concentrations, and three exposure durations: 48 h, 72 h, and 96 h. The beakers were covered in parafilm with several holes to minimize evaporation, and larvae were left to acclimate in the beakers in a temperature and light controlled chamber (16 °C, 24 h dark) until the start of exposure (8 dpf).

Exposures began at 8 dpf, approximately 24 h prior to hatch so that organisms hatched into respective treatments. Throughout the exposure, 50% media was changed daily, at which time hatching and any mortality were also recorded. At 48 h, 72 h, and 96 h of exposure (corresponding to 10, 11, and 12 dpf), three larvae were removed from each replicate to perform behavioral testing. Ten remaining larvae from the same beaker were euthanized on ice, snap frozen in liquid nitrogen, and stored in −80°C for downstream quantitative PCR (section 2.5.4).

2.5.3 Behavioral assessment of bifenthrin-exposed larvae—At the age of interest (10 dpf, 11 dpf, or 12 dpf), three larvae from each technical replicate of each exposure treatment were carefully placed into a well on a 12 well plate, containing 2 mL of water with target bifenthrin concentrations. Each plate was randomized such that it contained three larvae from each treatment. The larvae were allowed to adjust to the plate conditions for at least 1 h before the initiation of behavioral testing. The behavioral LD cycle

assays were conducted as previously described in section 2.2 (n= 6 plates, biological replicates n=18 larvae per treatment).

Total distance moved and velocity were collected for the bifenthrin-exposed larvae. The measured velocities were binned by speed into several categories including cruising (≥ 5 mm/s and ≤ 20 mm/s), bursting (≥ 20 mm/s), and freezing (≤ 5 mm/s). These speed categories were chosen to reflect categories measured in previous studies using zebrafish and fathead minnows (Steele et al., 2018). The categories were measured by duration (second per each minute at that speed) as well as frequency (number of times the larvae reached that speed per minute). To increase visual clarity while presenting multiple parameters having different units (e.g. mm/s, counts, and s), Z-score is presented in the figure only (Figure 3B), normalized to vehicle control. The calculation of Z-score was conducted using the following equation: Z-score = $(x - \mu) / \sigma$, where x= value, μ= mean, σ=standard deviation.

2.5.4 Gene expression of bifenthrin-exposed larvae

2.5.4.1 Primer Design: The genes chosen for quantitative polymerase chain reaction (qPCR) analysis represent several functional categories relevant to previous known outcomes of exposure to bifenthrin in teleosts, including the mTOR signaling pathway, neurological development and differentiation, and biotransformation (Table 1). Genes involved in the mTOR signaling pathway included the mechanistic target of rapamycin (*mtor*), DEP domain-containing mtor-interaction (*deptor*), Late endosomal/lysosomal adaptor, mtor activator 4 (*lamtor4*), Rapamycin-insensitive companion or mtor (*rictor*), Ras homolog enriched in the brain (*rheb*), and Inositol-triphosphase receptor type 3 (*itpr3*). Neurological development and differentiation genes include the Protoonco-gene c-fos (*cfos*), and Neurogenic differentiation factor 1 (*neurod1*). Biotransformation genes include ATP-binding cassette sub-family a member 2 (*abca2*), and Cytosolic sulfotranferase 3 (*sult1a3*).

Delta smelt sequences were obtained from a previously annotated transcriptome (Jeffries et al. 2016). Primers were designed using the Roche Assay Design Center (Roche Life Sciences, Indianapolis, IN, USA) and adjusted using Primer Express to more appropriately fit the parameters of the instrumentation used. Primer efficiencies were tested on a mixed pool of samples diluted in series.

2.5.4.2 Quantitative Polymerase Chain Reaction (qPCR): Total mRNA was extracted from whole larvae (10 individuals pooled) using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). The extracted RNA was tested for quality and concentration (ng μL^{-1}) using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Samples were accepted as being of sufficient quality having 260/280 and 260/230 ratios between 2 to 2.13, and 1.75 to 2.3, respectively. A subset of these samples was assessed using a 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA), which confirmed RIN scores ranging from 6.9 to 10. Complementary DNA (cDNA) was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) according to the user's manual. Primer efficiencies were tested on a mixed pool of samples diluted in series.

Quantitative PCR was conducted using SYBR® Green PCR Master Mix (Qiagen QuantiTect®). Reactions consisted of 6 biological triplicates and 3 technical replicates per

treatment, conducted in 384-well plates using the 7900HT Fast Real-Time PCR Systems by (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Amplification data were analyzed using Sequence Detection Systems software (SDS v2.4; Applied Biosystems). GeNORM algorithm was used in qbase+ software (Biogazelle, Zwijnaarde, Belgium) to assure stability of reference gene expression across samples. Reference genes included *actb1*, *gadph*, and *rpl7* (Table 1).

2.6 Statistics

2.6.1 Delta smelt larvae behavior—For all behavior, parameter (total distance moved or velocity) was binned by minute. To measure differences in movement in the LD cycle assays, averages were calculated and compared within the tests per cycle. Cycle refers to the periods of dark or light including Dark 1, Light 1, Dark 2, Light 2, and Dark 3. For all behavioral analysis tests including treatments (housing conditions or bifenthrin exposure), treated fish were compared to control fish only from the same batch (each batch containing a clutch from 2 females and 2 males, outlined in Fish Care, section 2.1), and all tests were compared only within the same age group (10, 11 or 12dpf).

For all behavior tests, a nonparametric Kruskal-Wallis analysis of variance was run to test the effects of cycle, housing condition, or bifenthrin treatment on the measured parameter using the kruskal_test function in R (Kassambara, 2020). As a post-hoc analysis for the LD cycle assay development, Dunn's multiple comparisons test was conducted to compare distance moved between cycles (at $\alpha < 0.05$ with Bonferroni p-value adjustment), using the dunn_test function in R (Kassambara, 2020). As post-hoc analysis for housing condition and light-dark preference tests, a Student's *t*-test was conducted to compare distance moved or portion of time spent within cycles (at $\alpha < 0.05$), using the t_test function in R (Kassambara, 2020). As post-hoc analysis for bifenthrin exposure experiments, contrasts were assessed via emmeans multiple comparison test in R (Lenth, 2019), using the contrast method trt.vs.ctrl to compare vehicle control with treatments 2, 10, and 100 ng/L bifenthrin ($\alpha < 0.05$). The p-value was adjusted using the dunnetx method (Dunnett's test) for 3 tests (Vehicle versus 2, 10, and 100 ng/L bifenthrin).

2.6.2 Gene expression of bifenthrin-exposed larvae—To determine differences in expression of each gene between bifenthrin concentrations, a nonparametric Krukal-Wallis analysis of variance test was run to test the effects of treatment on gene expression, using the kruskal_test function in R (Kassambara, 2020). As a post-hoc analysis, contrasts were assessed via emmeans multiple comparison test in R (Lenth, 2019), using the contrast method trt.vs.ctrl to compare vehicle control with treatments 2, 10, and 100 ng/L bifenthrin. The p-value was adjusted using the dunnetx method (Dunnett's test) for 3 tests (Vehicle versus 2, 10, and 100 ng/L bifenthrin). Graphs with untransformed values, analyzed via the $Log_2^{(-\Delta\Delta CT)}$ method are shown in the Supplementary material (Supplemental Figures 3 and 4).

To measure expression in a dose-responsive manner, the data were analyzed using regression analyses to fit concentration-effect curves based on an approach applied in Frank et al.

(2019). A maximum likelihood estimate (MLE) approach was used to evaluate whether non-monotonic curves were a better fit to the data than a null (intercept-only) model. Five different concentration-effect curves (linear regression, quadratic, sigmoidal, 5-parameter unimodal, and 6-parameter unimodal) were tested to fit responses of all three concentrations and vehicle control. A maximum likelihood ratio test was used to examine whether each curve provided a better fit than an intercept- only null model with a significance level of $\alpha < 0.05$. All calculations for the concentration-effect curves were performed using fold-change values.

3. RESULTS

3.1. LD cycle assay development and light period housing effects

At all three developmental timepoints, total distance moved in the light period was significantly higher in periods Light 1 and Light 2, compared to the dark periods (Figure 1A) ($p < 0.0001$, Dunn's test). At 10 dpf, larvae housed in 12:12 light:dark moved significantly more during the Dark 2 period ($p < 0.01$, Student's *t*-test) (Figure 1B). At 11 and 12 dpf, no difference in movement was found between differentially housed larvae in Light 1 or 2; however, those housed in 12:12 light:dark moved significantly more in Dark 2 and Dark 3, and in all dark periods at 12 dpf (Figure 1B) ($p < 0.05$, $p < 0.0001$ (11 dpf); $p < 0.01$, $p < 0.05$, $p < 0.0001$ (12 dpf), Student's *t*-test). A timeline representation of each minute is shown in Supplementary material (Figure S1).

3.2 Light-Dark preference test

No consistent pattern of preference was observed in larvae during the LD cycle assay. At 11 dpf, larvae spent more time in the covered area than the open during Dark 1 ($p < 0.05$, Student's *t*-test), and more time in the covered area during Light 2 at 12 dpf ($p < 0.01$, Student's *t*-test) (Figure 2A).

In an extended 20 min Light period and 20 min Dark period, larvae exhibited a consistent pattern of preference for lighted areas during the Light period (Figure 2B). At 10, 11, and 12 dpf, the larvae spent significantly more time in the lighted area than the covered area ($p < 0.0001$, Student's *t*-test) (Figure 2B). Conversely, at 10 and 12 dpf, larvae spent significantly more time in the covered area during the dark period ($p < 0.001$, $p < 0.01$, Student's *t*-test). At 11 dpf, the larvae spent significantly more time in the lighted area than the covered area during dark period ($p < 0.05$, Student's *t*-test).

3.3 Behavioral assessment of bifenthrin-exposed larvae

Larvae exposed to environmentally relevant concentrations of bifenthrin for 96 h exhibited increased movement (distance) in the LD cycle assay (Figure 3). At 96 h of exposure, 10 ng/L and 100 ng/L treatments of bifenthrin caused the smelt larvae to move significantly more during Light 2 in comparison to vehicle control (10 ng/L, $p < 0.01$; 100 ng/L, $p < 0.05$, Dunnett's test). Exposure to 10 ng/L bifenthrin caused increased movement compared to vehicle control in all dark periods ($p < 0.05$, $p < 0.05$, $p < 0.01$, Dunnett's test). A timeline representation of each minute is shown in Supplementary material (Figure S2).

Although larvae exposed to 2 ng/L bifenthrin showed no difference in total distance moved, differences in velocity were observed (Figure 3B). During Light 2 and Dark 3, larvae exhibited significant increases in velocity ($p < 0.05$, Dunnett's test), and a significant increase in freezing frequency during Dark 3 in comparison to vehicle control ($p < 0.05$, Dunnett's test).

During all dark periods, larvae exposed to 10 ng/L bifenthrin exhibited a significantly increased frequency of bursting and cruising events ($p < 0.05$, $p < 0.05$, $p < 0.01$, Dunnett's test). Larvae exposed to 10 ng/L bifenthrin also displayed increased velocity and decreased freezing durations ($p < 0.05$, Dunnett's test) during Dark 2, and increased velocity, cruise duration, and bursting duration during Dark 3. During Light 2, larvae showed increased velocity, cruising duration, and decreased freezing duration ($p < 0.01$, $p < 0.05$, $p < 0.05$, Dunnett's test). Larvae exposed to 100 ng/L bifenthrin displayed increased cruising frequencies during Dark 1 and Dark 2 ($p < 0.05$, Dunnett's test). During Light 2, larvae increase velocity, cruising duration, cruising frequency, bursting duration, bursting frequency ($p < 0.05$, Dunnett's test).

3.4 Gene expression of bifenthrin-exposed larvae

Quantitative PCR revealed that genes that function in biotransformation, *abca2* and *sult3a*, were not differentially expressed relative to vehicle control. In the mTOR pathway, *itpr2* was significantly upregulated at 2 ng/L exposure at 96 h ($p < 0.05$, Dunnett's test) (Figure 4A). As it relates to neurogenesis, *cfos* was significantly downregulated at 2 and 10 ng/L exposure at 72 h ($p < 0.05$, Dunnett's test) (Figure 4B).

Two genes in the mTOR pathway showed concentration-dependent expression, although no significant changes compared to vehicle control in expression were observed. At 48 h, for *deptor* there was a significant negative relationship between expression and dose ($p < 0.05$, MLE) (Figure 4A). At 72 h, *lamtor* significantly fit a negative quadratic curve of expression by dose ($p < 0.05$, MLE) (Figure 4A). Both genes specific to neuron growth and differentiation fit a quadratic curve of expression at 72 h of exposure; *cfos* fitting a negative quadratic curve ($p < 0.01$, MLE), while *neurod1* fit a positive quadratic curve ($p < 0.05$, MLE) (Figure 4B). Of the genes chosen for biotransformation, *abca2* fit significantly to a positive quadratic curve of expression at 96 h of exposure ($p < 0.05$, MLE) (Figure 4B).

4. DISCUSSION

In this study we adapted a behavioral assay that has been validated for use on other teleost larvae species, and confirmed its efficacy for use with larval Delta smelt, and show they have a preference to move towards lighted areas. We utilized the behavioral test to evaluate the impact of exposure to bifenthrin, and showed increased hyperactivity in larvae exposed to bifenthrin for 96 h.

4.1 LD cycle assay development

The endangered Delta smelt are highly sensitive to handling, thus not comparable to robust model teleost species such as zebrafish and fathead minnow commonly used in toxicity

testing (EPA, 2002). The adapted LD cycle behavioral assay is therefore advantageous for this species in that it can greatly limit disruption of the larvae during testing, potentially allowing more subtle sublethal effects to be observed. To limit disruption to the larvae, the LD cycle assay can be planned such that a 96 h exposure is possible within the developmental window of which the test is consistent. Exposures during early development can be achieved by initiating exposure at 8 dpf (pre-hatch), ending the test prior to feed requirements. Exposing the individuals pre-hatch is also advantageous in that it eliminates the need to handle newly hatched Delta smelt larvae via pipette. Because we observed significant differences in total distance moved between cycles, we can use this as a metric by which to measure hyper- or hypoactivity within each cycle.

4.2 Light period housing effects on the LD cycle assay

Previous toxicology and developmental experiments have been conducted on Delta smelt larvae using housing with varying light conditions. For example, a range of 8:16 h light:dark, to 24 h dark have been used (Connon et al., 2009; Romney et al., 2019). Current practices at the FCCL use a 9–10 μmol/m^2/s (~740 Lux) for embryo and larval rearing with a continuous 24 h ambient light (and 0 dark cycle). Because the circadian rhythm is linked to hormonal and neuronal development, it is important to consider how pre-test housing conditions may impact the outcome. A study by Villamizar et al. (2014) suggested that photoperiod can greatly affect zebrafish larval survival, specifically that 24 h dark housing resulted in 100% mortality, whereas those transferred to a light:dark cycle had an increase in survival. Additionally, those kept in 24 h red lighting (visible light wavelengths) exhibited reduced feeding activity, and resulted 100% mortality, suggesting that lighting conditions may contribute to larval health via ability to find and ingest food. It is recommended that fathead minnow be reared in a photoperiod of 16:8 h light:dark (EPA, 2002). Interestingly, there is no formal recommendation on photoperiod for zebrafish larval housing in *The Zebrafish Book* (the most widely used recommendation source for zebrafish care); however, many laboratories house their larvae in a 14:10 h light:dark photoperiod (Westerfield and ZFIN, 2000). Considering the stimulus in the LD cycle assay is exposure to bright light, we sought to determine suitable lighting conditions for housing Delta smelt larvae, in relation to their responsiveness to light stimuli. Our data indicate that differences in photoperiod (larvae housed in 24 h dark or 12:12 h light:dark) minimally alter the outcome of the LD cycle behavioral assay. Particularly, the decreased movement in the dark periods of larvae housed in 12:12 h light:dark conditions is to note for those designing behavioral experiments using this and other non-model organisms, as it will likely aid assay optimization.

4.3 Light-Dark preference test

Delta smelt larvae, reportedly, swim towards light sources (Lindberg et al., 2013). Both zebrafish and fathead minnow larvae preferentially move towards the light, and both lose this behavioral trait as juveniles and adults (Mueller and Neuhauss, 2002; Steenbergen et al., 2011; Vignet and Parrott, 2017). Because the Delta smelt larvae exhibit such consistent behavior when exposed to bright light, we investigated a potential cause for the increase in movement during the light period. We hypothesized that the increase in movement around the well was due to the larvae preferentially moving towards the light.

The wells were lit from beneath, thus the direction of light was mostly ubiquitous within the chamber, potentially causing the circular motion of the larvae around the well. Because modified motion in response to the light stimulus is the ultimate outcome of the LD cycle assay, it is important to determine their behavior when given an option to remain in either dark or light areas (preference test). When the larvae were evaluated for light-dark preference during LD cycle assay, no consistent differences were found in preference between portion of time spent in the lighted (open) or dark (covered) areas. It is possible that a short duration (5 min light period) does not allow the larvae enough time to determine which side is preferred, as they may initially be startled by the initiation of the light. This can be seen during the first minute of light exposure as a slightly increased amount of total distance moved in comparison to each other minute in the 5 min light periods. When the test was modified to include an extended 20 min light and sequential 20 min dark period, we observed significant differences in time spent in each open or covered areas; the larvae spending more time in the open area during the light period at each developmental stage (10, 11, or 12 dpf).

Preference for lighted areas has been observed in other teleost larvae (Burgess et al., 2010; Vignet and Parrott, 2017), and there are several hypotheses as to why some larvae preferentially move towards visible light. One hypothesis suggests larval movement towards lighted areas is driven by foraging behavior, as their food source would typically be found at the surface of the water in a more lighted area (Burgess and Granato, 2007). Another hypothesis suggests larvae preferentially move towards the light so as to more effectively camouflage from predators (Abrahams, 2005). Considering the Delta smelt larvae are translucent upon hatching, the predation avoidance hypothesis may be supported.

Understanding the mechanism(s) behind this preference would strengthen the interpretation of the LD cycle assay data. It is important to recognize that preference towards light may not be a contributor to increased movement in the LD cycle assay for Delta smelt larvae. For example, zebrafish larvae exhibit preference towards light when given a choice (Burgess and Granato, 2007), but decrease movement in lighted conditions in light-dark phase cycling assays, which is attributed to a passive fear response (Rennekamp et al., 2016). Additional studies are needed to be conducted in order to definitively determine why this preference in the 20 min extended Light-Dark test exists, and if fear circuits are responsible for increased movement of Delta smelt larvae in the Light periods during the LD Cycle assay.

4.4 Behavioral assessment of bifenthrin-exposed larvae

We utilized the LD Cycle assay to test effects of exposure to environmentally relevant concentrations of a neurotoxic pesticide that is ubiquitously present in the SFBD. The test revealed that at 96 h of exposure, all concentrations of aqueous bifenthrin exposure caused hyperactivity in larval Delta smelt either by increasing total distance moved or velocity. At 96 h of exposure to 10 ng/L and 100 ng/L bifenthrin, the larvae showed an increase in velocity and total distance moved, suggesting they moved faster for longer periods of time, contributing to the increase in total movement. Larvae exposed to 2 ng/L bifenthrin still exhibited an increase in velocity, but no increase in total movement was observed. Because the increase in velocity is also paired with increased frequency of freezing events, this

indicates the larvae may make fast movements for short periods of time, starting and stopping more frequently.

Of importance to note is the current limitation of interpretation of behavioral changes in Delta smelt larvae in this particular assay in terms of variability and magnitude. We have observed high variability between spawned batches of Delta smelt. This could be due to differences in domestication indices associated with rearing this endangered species. However, behavioral outcomes across teleost larval spawns can often be extremely variable (Dach et al., 2019). For Delta smelt, we account for this in our experimental design by only using larvae from a single batch for each exposure. The variability in movement within the LD cycle assay increases with age, and significant differences in movement between cycles are potentially lost past 12 dpf (unpublished data), suggesting there is an optimal developmental stage at which to perform the test. Further studies would be needed to confirm the age at which this behavior is lost.

Additionally, the changes in total distance moved of treated fish compared to control reached approximately 65% increase at the largest change (10ng/L at 96h during Light 2). Further testing with compounds known to elicit hyper or hypo-activity in other teleost species, such as pentylenetetrazol or midazolam respectively, would allow a more thorough understanding of the limits of the test regarding the categorization of an extreme or minute reaction. Although these compounds are not ecologically relevant in terms of potential exposure to wild Delta smelt, the establishment of behavioral standards would increase the value of interpretation of behavioral results.

Bifenthrin-induced hyperactivity poses potential risk to the Delta smelt, and other fish populations. For example, hyperexcitability or inability to complete normal escape behavior could allow the larvae to be more susceptible to predation in an ecological context. Pyrethroids, such as esfenvalerate, have been shown to be able to increase predation risk in fish larvae, particularly increasing predation risk in Fathead minnow larvae when exposed to 0.455 and 1.142 μg/L (Floyd et al., 2008).

Because bifenthrin is prevalent in the water system which the Delta smelt inhabits (Weston et al., 2019), it provides further weight of evidence that contaminants, at concentrations detected in the system, are likely playing a role in the decline of this species, supporting the conclusions of a review by Connon et al. (2019). Exposure has the potential to decrease larval fitness, potentially impact neurodevelopment, increase risk of predation and impact the capacity for prey capture.

Teleost larval stages are sensitive to contaminants representing a particularly vulnerable life stage, with elevated risks associated with compound accumulation (e.g. via yolk-sac absorption) and early developmental disturbances which have the potential of resulting in immediate, or long-term behavioral abnormalities, should the organisms survive early exposure. The larval fish yolk-sac provides a highly lipophilic substrate for contaminants such as bifenthrin to penetrate and/or adsorb to, as well as accumulate. Studies have found the half-life of bifenthrin to be 15.9 or 38.5 h in zebrafish larvae exposed to 2 or 20 μg/L, respectively (Tu et al., 2014). Bifenthrin exposure at this sensitive developmental stage could

have lasting effects both in terms of direct impact on neurogenesis, as well as latent long term or multigenerational effects from bioaccumulation (Major et al., 2020). Our prior studies on zebrafish have found delayed effects of bifenthrin exposure, causing hyperactivity in photomotor behavioral tests at 19 dpf, in fish that were exposed to 1 and 10 ng/L from 2 to 5 dpf (Frank et al., 2018). Environmentally relevant concentrations of bifenthrin have also been shown to impair predator avoidance in Inland silversides (Frank et al., 2019). Bifenthrin exposure has also been reported to cause behavioral deficits in adult rats (Syed et al., 2018), including motor activity, motor incoordination, and cognitive impairment, suggesting that bifenthrin could affect a mature brain differently to a developing brain.

4.5 Gene expression of bifenthrin-exposed larvae

Transcriptomic responses can be a powerful approach to evaluate organismal responses to environmental stressors (Connon et al., 2019). Because the concentrations used in this study reflect those found in the environment, and are much lower than those typically used in broad range toxicology studies, the patterns of gene expression observed serve to inform modes of action in a realistic setting. Measured changes in expression occurring at picomolar bifenthrin concentrations suggests impacts on behavior, and potentially long-term effects associated with neurodevelopment.

Across functional categorization, most gene expression responses did not result in a significant dose-response pattern, and those significant were often a non-monotonic (quadratic) response with significant increase or decreases at only the low or mid concentration (Figures 4A and 4B). Non-monotonic responses in gene expression in response to bifenthrin exposure has previously been observed in other teleost species (Brander et al., 2016; Frank et al., 2018). Non-monotonic responses may arise for numerous reasons, one potential explanation being increased ability to metabolize lower concentrations of bifenthrin, of which the metabolites are more potent than the parent compound itself (Brander et al., 2016).

4.5.1 Neurodevelopment—At 72 h of exposure, *neurod1* expression fit significantly to a positive quadratic curve by concentration, and *cfos* expression fit significantly to a negative quadratic curve by concentration. Functioning as a transcription factor that promotes differentiation in new neurons and expressed spatiotemporally, *neurod1* has been used as a marker for neuronal growth (Horzmann and Freeman, 2016). Although the pattern of *neurod1* expression in the brain of Delta smelt is not characterized, in zebrafish it has been shown to decrease in expression levels after 72 hpf; following rapid development of the brain (Wullimann and Mueller, 2004). Because expression of *neurod1* is correlated with neuronal growth, the dose-dependent differences observed in Delta smelt at a single developmental timepoint may suggest dose-dependent altered neuronal growth. Additional experiments would be necessary to confirm altered neuronal growth in Delta smelt larvae following bifenthrin exposure. As a marker of neuronal activation, induction of *cfos* expression is known to occur following noxious stimulation or tissue injury (Gao and Ji, 2009). In zebrafish, increases in cfos expression have been found to occur following exposure to pesticides. Specifically, cypermethrin, a pyrethroid pesticide, was found to increase cfos mRNA expression in adult zebrafish brain at 0.0024 μM for 96 h (Özdemir et

al., 2018). Because *cfos* induction has been observed paired with hyperactive behavior, we hypothesized the larval Delta smelt would show an increase in *cfos*. Surprisingly, a significant decrease in *cfos* was observed at 2 and 10 ng/L bifenthrin at 72 h of exposure (log_2 fold-change of −0.56 and −0.50, respectively). Perhaps this suggests the mechanism at which low doses of bifenthrin, which cause hyperactivity, occur in a manner other than that resulting in noxious stimulation or neuronal injury.

4.5.2 mTOR pathway—At 48 h of exposure, we observed a significant fit to a negative linear trend in *deptor* expression by concentration. Because *deptor* is present in both mTORC1 and mTORC2 complexes, we are unable to distinguish to which pathway this is relevant (Takei and Nawa, 2014). At 48 h, *rictor*, which binds only in the mTORC2 complex, exhibited a non-significant positive quadratic response by concentration. This increase in *rictor* paired with the significant linear decrease in *deptor* at 48 h could indicate a possible preference of mTORC2 pathway upregulation at 48 h of exposure in response to 2 and 10 ng/L.

In a pattern similar to that at 48 h of exposure, *rictor* exhibits a non-significant positive quadratic dose-response at 72 h of exposure. Additionally, expression of *lamtor4* fit significantly to a positive quadratic curve at 72 h. As with 48 h of exposure, this may indicate a possible impact on mTORC2 in response to exposure to 2 and 10 ng/L bifenthrin. Paired with a significant decrease in *cfos* at 72 h of exposure, this could potentially indicate a neurodevelopmental delay.

At 96 h of exposure, *itpr3* fit non-significantly to a positive quadratic dose-response, with a significant increase at 2 ng/L compared to vehicle control (log_2 fold-change of 0.60). Considering significant changes in behavior were found at 96 h at all concentrations, the increase in *itpr3* at this timepoint are notable. Because *itpr3* functions in intracellular calcium release, and bifenthrin has been found to alter calcium oscillations in cultured rodent neurons (Cao et al., 2014), we hypothesized bifenthrin would increase *itpr3* expression. An increase in expression of *itpr3* is not observed until 96 h. Because Ca^+ is an activator of the mTOR pathway, perhaps a latent response in mTOR activity would be observed after 96 h. More experiments assessing Delta smelt later in development after acute exposures during early development would be needed. A pattern of latent mTOR expression was reported by Frank et al. (2019), in Inland silversides exposed to 3 ng/L bifenthrin from 5–7dpf, which exhibited increased mTOR abundance later in development; at 21dpf.

4.5.2 Biotransformation—A cytosolic sulfotransferase that functions in the sulfonation of endogenous molecules as well as xenobiotics, *sult1a3* is found primarily in intestine and brain in humans (James et al., 2013). We observed a significant decrease in mRNA abundance of *sult1a3* at 10 ng/L at 72 h of exposure (log_2 fold-change of −0.26). Cytosolic sulfotransferases are known to be regulated by nuclear hormone receptors (Runge-Morris et al., 2013). Because pesticides, particularly pyrethroids, are known to cause disruption in hormone regulation (Brander et al., 2016), it is possible the observed decrease in mRNA expression could be attributed to hormonal disruptions. For example, because dopamine is a substrate for *sult1a3* (James et al., 2013), it is possible that less available dopamine due to the stress of exposure could result in a reduction of *sult1a3* transcription. Other studies using

bifenthrin have documented altered dopamine signaling. Specifically, Crago and Schlenk (2015) found a decrease in dopamine receptor 2A mRNA expression in brains of juvenile Rainbow trout (*Oncorhynchus mykiss*) at 96 h and 2-week exposure to 3.55 pM bifenthrin.

5. CONCLUSIONS

We optimized a behavioral test that leveraged the observed natural photomotor response of Delta smelt yolk-sac larvae. Delta smelt larvae are less motile in the dark, and are stimulated by, and increase motility under light conditions. Use of this assay confirmed that Delta smelt are significantly impacted by concentrations of bifenthrin that are detected in their spawning and rearing habitat. Concentrations as low as 2 ng/L resulted in increased velocity, paired with increased frequency of freezing events, and at concentrations above 10 ng/L, hyperexcitability in terms of total distance moved was observed. Within an ecological context, this is likely to impact their ability to forage as well as escape from predators. Transcriptomic data reveal potential impacts on neurodevelopment. The implementation of the described behavioral test, adapted from zebrafish to Delta smelt, to evaluate water quality at known spawning and rearing locations, may serve to inform conservation managers on risks associated with exposure. This could in turn provide a monitoring tool to guide and assess the efficacy of planned and ongoing habitat restoration geared toward supporting the conservation of numerous fish species in the SFBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Delta smelt larvae increase movement in light and decrease movement in dark.
  - Delta smelt larvae preferentially move towards lighted areas.
  - When exposed to bifenthrin for 96 h, Delta smelt larvae increase movement.

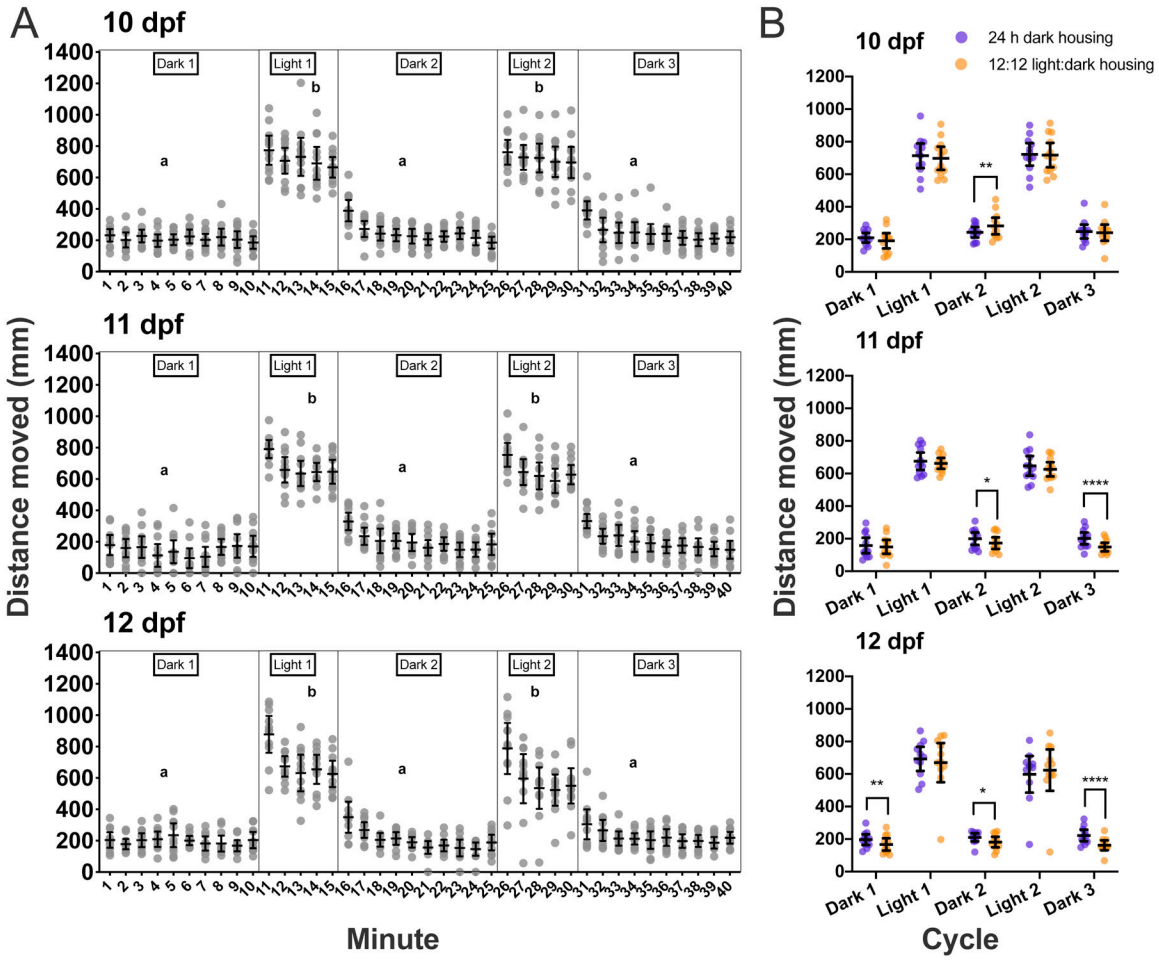

Figure 1. Total distance moved at 10, 11, or 12 dpf in the LD cycle assay and different housing conditions.

A) Total distance moved, binned by minute, of Delta smelt larvae at 10, 11, or 12 dpf. Individual points represent biological replicates (n=12 larvae), bars represent mean and 95% confidence interval. Compact letter display represents groups (mean total distance moved over entire cycle) significantly different from each other in Dunn's multiple comparisons test, $p<0.05$. **B**) Mean total distance moved over each cycle, of Delta smelt larvae at 10, 11, or 12 dpf, housed in either 24 h dark or 12:12 light:dark conditions. Individual points represent biological replicates (n=11–12 larvae), bars represent mean and 95% confidence interval. *$p<0.05$, **$p<0.01$ in Student's *t*-test.

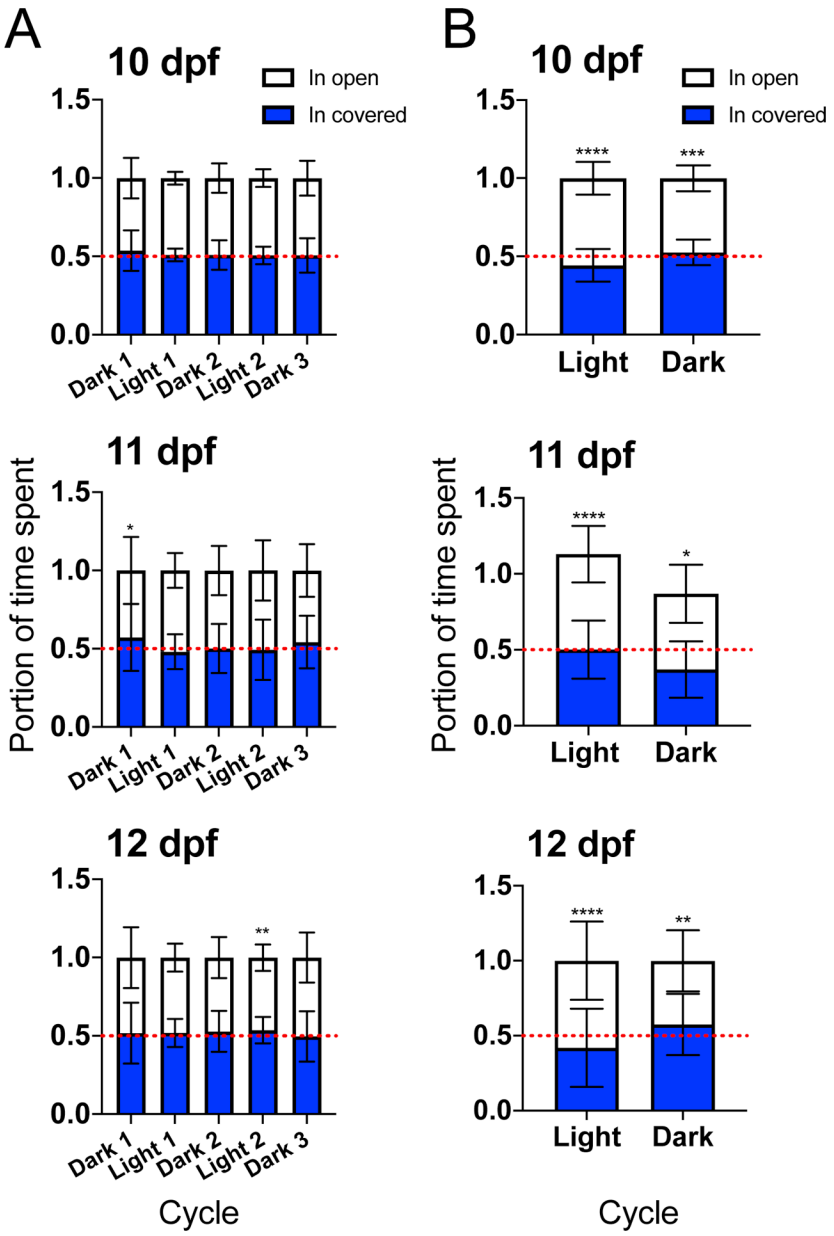

Figure 2. Preference for open or covered area during LD cycle assay or extended light or dark period, across 10, 11, or 12 dpf larvae.

A) Portion of time spent in either open or covered area during the LD cycle assay. Open bars represent mean portion of time spent in open area of arena (seconds recorded of larvae in the open area divided by seconds recorded of larvae in arena, binned by minute and averaged over cycle). Blue bars represent portion of time spent in covered area of arena (seconds recorded of larvae in the covered area divided by seconds recorded of larvae in arena, binned by minute and averaged over cycle). Error bars represent standard deviation of the mean. n=23–24 larvae, **p<0.01, ****p<0.0001 in Student's *t*-test. **B)** Average portion of time spent in either open or covered area of arena during either the 20 min light or 20 min dark period. Open bars represent mean portion of time spent in open area of arena (seconds recorded of larvae in the open area divided by seconds recorded of larvae in arena, binned by

minute and averaged over cycle). Blue bars represent mean portion of time spent in covered area of arena (seconds recorded of larvae in the covered area divided by seconds recorded of larvae in arena, binned by minute and averaged over cycle). Error bars represent standard deviation of the mean. n=19–36 larvae, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 in Student's *t*-test.

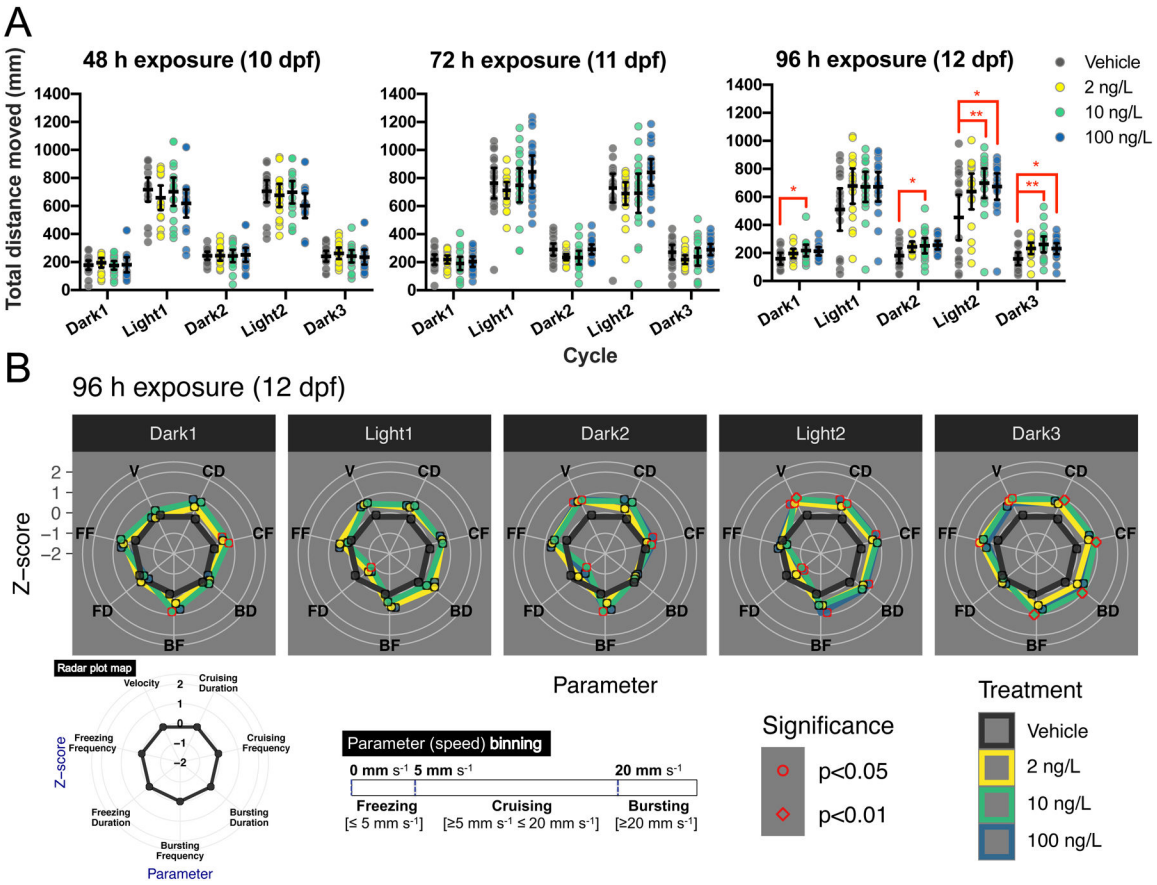

Figure 3. Movement in LD cycle assay during exposure to bifenthrin.

A) Mean total distance moved over each cycle, of Delta smelt larvae at 48 h, 72 h, or 96 h of exposure (which correspond to 10, 11, or 12 dpf). Individual points represent biological replicates (n=18), bars represent mean and 95% confidence interval. *p<0.05, **p<0.01, in Dunnett's test, comparing all treatments to vehicle control within each cycle. **B)** Velocity parameters over each cycle, of Dela smelt larvae at 96 h of exposure to bifenthrin. Parameters are defined as cruising (≥ 5 mm/s ≤ 20 mm/s), bursting (≥ 20 mm/s), and freezing (≤ 5 mm/s). Parameters included in the graph are Velocity (mm/s), Duration (time spent in the respective velocity range, s), and Frequency (number of times the larvae initiated/terminated movement in a respective velocity range, count number). The plotted circles are representative of the calculated Z-score (calculation below) of each parameter (across treatments), normalized to the vehicle control. Z-score is presented for visual purposes, normalized to vehicle control. n=18 larvae, (red circle outline) $p < 0.05$, (red diamond outline) $p < 0.01$ in Dunnett's test. Calculation: z-score = $(x - \mu) / \sigma$, where x=value, μ= mean, σ=standard deviation.

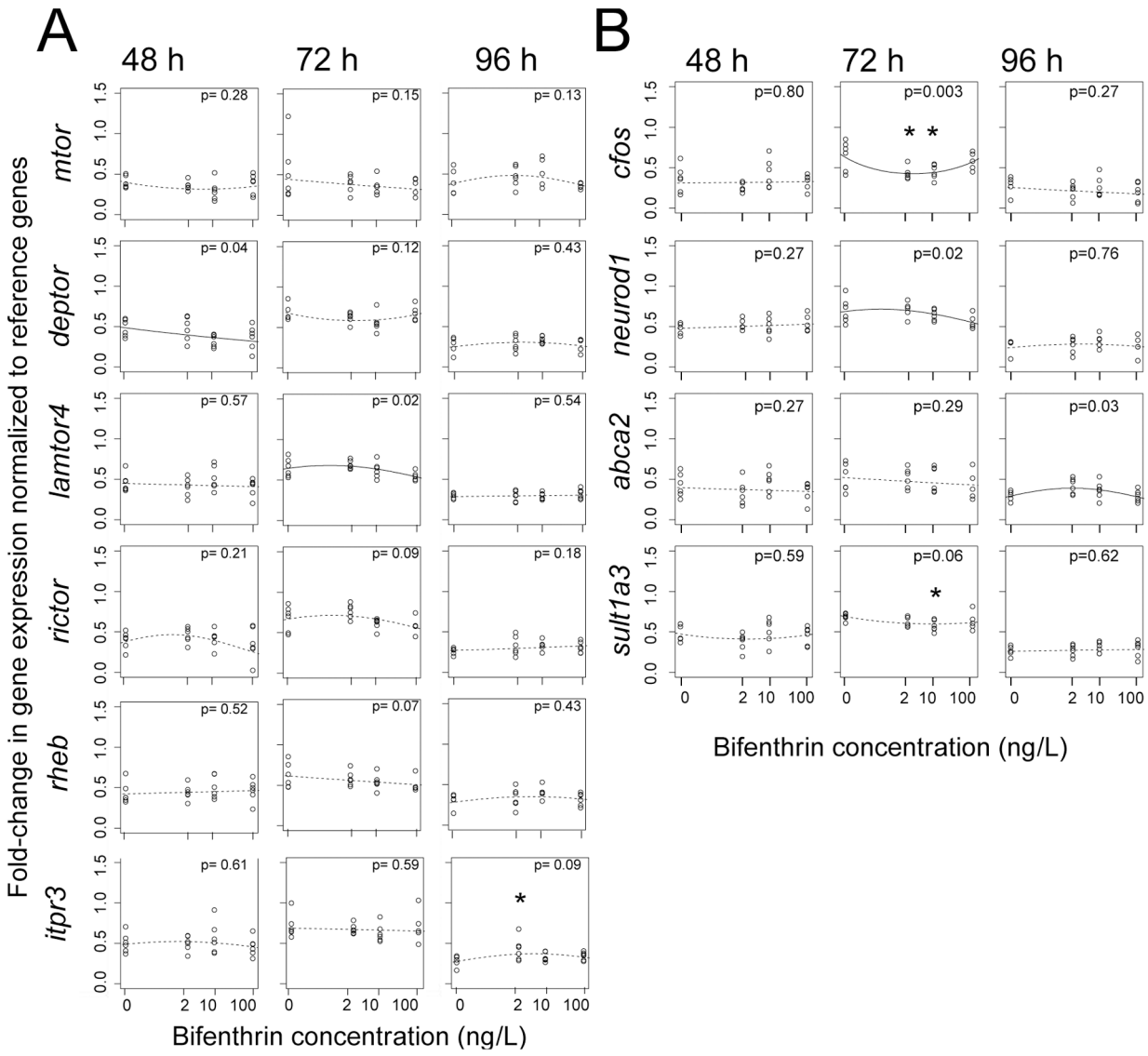

Figure 4. Changes in transcript abundance of genes in mTOR pathway, neurodevelopment, and biotransformation in Delta smelt larvae exposed to varying concentrations of bifenthrin for 48 – 96 h.

Each circle represents the fold change value of a single biological replicate, normalized to the average of the reference genes *actb1*, *gadph*, and *rpl7*. Genes relevant to mTOR pathway are shown in panel **A** and genes relevant to neurodevelopment and biotransformation are shown in panel **B**. Data are presented on a log10 X+ 0.05 axis. For each panel, the data was assessed being fit to five curves (linear, quadratic, sigmoidal, unimodal1, and unimodal2) using a maximum likelihood approach. The best fitting curve is shown in each panel. Curves shown as a solid line are significantly better fits than a null intercept-only model ($p<0.05$), curves shown as a dashed line are the best-fit of the five-curve option (lowest p-value), but not significantly better than the null model. P-values for each fit curve are shown in the panel. n=6, *$p<0.05$ in Dunnett's test in comparison to vehicle control.

Table 1.
Genes selected for transcriptomic analysis.

This table includes genes selected in order of functional category including the mTOR pathway, neurological development, and biotransformation.

Function	Gene Name	Gene code	Primer (5'-> 3')	%Efficiency
Reference Genes				
Reference	Beta-actin	*actb1*	F : tgccacaggactccata R : catcggcaacgagaggtt	104.11
Reference	Glyceraldehyde-3-phosphate dehydrogenase	*gadph*	F : tccacgagaaagacccaact R : cacgccagtagactcaacca	96.11
Reference	60S ribosomal protein L7	*rpl7*	F : ccgtacagcccgcaaagtt R : tgaagtcaatggccagtttgg	97.02
Genes of Interest				
mTOR pathway	Mechanistic target of rapamycin	*mtor*	F : gacaccatgggctacctgcta R : atctccgtcctcacggcc	104.11
mTOR pathway	DEP domain-containing mtor-interaction	*deptor*	F : cctcgtacaggtgtcactccatc R : tgcagtagctgctgtcgcttt	100.37
mTOR pathway	Late endosomal/lysosomal adaptor, mtor activator 4	*lamtor4*	F : cagtgaagatggcgtgcttg R: cagcagagctaatccgacatcc	90.61
mTOR pathway	Rapamycin-insensitive companion of mtor	*rictor*	F : cgtacatgggggtgaagtgg R : tctgcctgtactctgaggtgtcc	102.38
mTOR pathway	Ras homolog enriched in brain	*rheb*	F : gttatcagctgtgaagagggtaagg R : gAacacctctacggctgtctgg	94.27
mTOR pathway	Inositol –triphosphate receptor type 3	*itpr3*	F : acctcccagaaccagtctgct R : gccccctcatgcagtagaag	99.68
Neurodevelopment	Proto-oncogene c-fos	*cfos*	F : gccaacaccatatccacctcc R : ccaggtcggacatcttgactg	109.91
Neurodevelopment	Neurogenic differentiation factor 1	*neurod1*	F : gggtgcctgcagctgaat R : agtagggatgcgcagcaaa	91.52
Biotransformation	atp-binding cassette sub-family a member 2	*abca2*	F : tcgaggaccactgcgaaac R : gacaaagggaatggcagtcg	91.82
Biotransformation	Cytosolic sulfotransferase 3	*sult1a3*	F : ttatagccacatatcccaaagcag R : caaagggaccaagttctgacg	103.52