HHS Public Access

Author manuscript

*Aquat Toxicol*. Author manuscript; available in PMC 2019 July 01.

Published in final edited form as:

*Aquat Toxicol*. 2018 July ; 200: 50–61. doi:10.1016/j.aquatox.2018.04.003.

# Bifenthrin causes transcriptomic alterations in mTOR and ryanodine receptor-dependent signaling and delayed hyperactivity in developing zebrafish (*Danio rerio*)

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## Abstract

Over the last few decades, the pyrethroid insecticide bifenthrin has been increasingly employed for pest control in urban and agricultural areas, putting humans and wildlife at increased risk of exposure. Exposures to nanomolar (nM) concentrations of bifenthrin have recently been reported to alter calcium oscillations in rodent neurons. Neuronal calcium oscillations are influenced by ryanodine receptor (RyR) activity, which modulates calcium-dependent signaling cascades, including the mechanistic target of rapamycin (mTOR) signaling pathway. RyR activity and mTOR signaling play critical roles in regulating neurodevelopmental processes. However, whether environmentally relevant levels of bifenthrin alter RyR or mTOR signaling pathways to influence neurodevelopment has not been addressed. Therefore, our main objectives in this study were to examine the transcriptomic responses of genes involved in RyR and mTOR signaling pathways in zebrafish (*Danio rerio*) exposed to low (ng/L) concentrations of bifenthrin, and to assess the potential functional consequences by measuring locomotor responses to external stimuli. Wildtype zebrafish were exposed for one, three and five days to 1, 10 and 50 ng/L bifenthrin, followed by a 14 d recovery period. Bifenthrin elicited significant concentration-dependent transcriptional responses in the majority of genes examined in both signaling cascades, and at all time points

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examined during the acute exposure period (1, 3, 5 days post fertilization; dpf), and at the post recovery assessment time point (19 dpf). Changes in locomotor behavior were not evident during the acute exposure period, but were observed at 19 dpf, with main effects (increased locomotor behavior) detected in fish exposed developmentally to the bifenthrin at 1 or 10 ng/L, but not 50 ng/L. These findings illustrate significant influences of developmental exposures to low (ng/L) concentrations of bifenthrin on neurodevelopmental processes in zebrafish.

**Keywords**

$Ca^{2+}$-dependent signaling; fish behavior; insecticide; neurodevelopment; pesticide; pyrethroid

## 1. Introduction

Since the production of photostable pyrethroids in the 1970s, pyrethroid insecticides have been increasingly employed for insect control over the last four decades (Schleier III and Peterson, 2011), and they now represent the fourth largest group of insecticides used worldwide (Kuivila et al., 2012; Brander et al., 2016a). Moreover, in the past decade, bifenthrin, a broadly used pyrethroid, has been increasingly employed in urban areas (Jiang et al., 2012; Weston and Lydy, 2012; Saillenfait et al., 2015). As a result, there is the potential for high influx of bifenthrin into surface waters, particularly following heavy rains. Bifenthrin is commonly detected in surface waters in the low ng/L range (Weston and Lydy, 2012), although concentrations as high as 106 ng/L have been measured. Additionally, bifenthrin is the most frequently detected pyrethroid in river sediments in North America and Australia (Nowell et al., 2013; Allinson et al., 2015), and it has the potential to bioaccumulate in fish tissues (Munaretto et al., 2013). The latter is of particular concern because fish are considered more vulnerable to pyrethroid toxicity than mammals (Glickman and Lech, 1982).

Pyrethroids are classified as type I and type II, as defined by the absence or presence of an α-cyano-3-phenoxybenzyl moiety, respectively (Soderlund, 2012). This difference in chemical structure results in distinctive toxicologic effects: type I pyrethroids cause tremors or convulsions, while exposure to type II pyrethroids predominantly causes choreoathetosis (Casida and Durkin, 2013). Bifenthrin is a type I pyrethroid that binds to the voltage-gated sodium channels in insects to prolong the action potential in nerves; it also interacts with voltage-gated sodium channels of vertebrates (Mukherjee et al., 2010; Soderlund, 2012). More recently, pyrethroids have also been shown to affect calcium signaling via interactions with voltage-gated calcium ion channels (Soderlund, 2012), and exposure to nanomolar concentrations of bifenthrin has been shown to dysregulate $Ca^{2+}$ homeostasis in neurons cultured from developing rodent brain (Cao et al., 2014). Calcium signaling regulates diverse neurodevelopmental processes (Berridge et al., 2000; Lohmann, 2009), and perturbation of calcium signaling in the developing brain has been linked to deficits in neurobehavior (Gargus, 2009; Stamou et al., 2013).

Whilst there is a rich literature addressing the role of intracellular calcium signaling in fish models, particularly in goldfish (Johnson and Chang, 2002; Sawisky and Chang, 2005), the

effects of bifenthrin on calcium signaling in fish has not been previously addressed. Therefore, a main objective of this study was to assess the effects of bifenthrin on the transcriptional profile of calcium-dependent signaling molecules, in particular, ryanodine receptor (RyR) and mTOR-dependent signaling molecules, in developing fish. RyRs are calcium-dependent calcium release channels embedded in the endoplasmic reticulum that regulate calcium-dependent signaling in neurons, and their function is critical to normal neurodevelopment (Pessah et al., 2010). The mTOR signaling pathway is also critical for normal neurodevelopment (Kumar et al., 2005; Lee et al., 2011; Bowling et al., 2014; Tang et al., 2014), and it is activated by increases in intracellular calcium (Zhang et al., 2012). Both RyRs (Mackrill, 2012) and mTOR signaling molecules (Hall, 2008) are conserved throughout the eukaryotic kingdom. We recently reported transcriptional alterations in key genes of both the RyR and mTOR signaling pathways in developing zebrafish exposed to μM concentrations of polychlorinated biphenyl (PCB) 95 (Frank et al., 2017). PCB 95 is an environmental contaminant known to interfere with neurodevelopment in mammalian systems by modulating calcium-dependent signaling via RyR-dependent mechanisms (Wayman et al., 2012b; Wayman et al., 2012a). But whether classes of environmental contaminants other than PCBs that also alter calcium influx, such as bifenthrin, similarly alter the transcriptional profile of RyR and mTOR signaling molecules, and whether such transcriptional changes are associated with altered phenotypes in fish is not known.

To address these questions, we exposed wildtype zebrafish to low (ng/L) concentrations of bifenthrin during early development. Previous studies have examined both acute (Jin et al., 2009) and developmental toxicity (Shi et al., 2011; Tu et al., 2016) of bifenthrin in zebrafish, but there has been no study examining potential neurodevelopmental effects following exposure to picomolar concentrations of pyrethroids. Since the effects of developmental exposures to neurotoxic chemicals can manifest at later life stages (Levin et al., 2003), zebrafish were assessed for transcriptional and behavioral effects immediately following the cessation of bifenthrin exposure at 5 days post-fertilization (dpf), and at 19 dpf, 14 d after exposure ended.

## 2. Materials and Methods

### 2.1. Chemicals

Bifenthrin (CAS# 82657-04-3, purity > 98 %) was purchased from Chem Service (West Chester, PA, USA).

### 2.2. Fish husbandry and spawning

All research involving zebrafish, fish husbandry and spawning were performed in accordance with UC Davis Institutional Animal Care and Use Committee (IACUC) protocol #17645. Adult wild-type, Tropical 5D zebrafish (*Danio rerio*) were kept in 2.8 L tanks at a density of 5–7 fish per L at 28.5 ± 0.5°C on a 14 h light:10 h dark cycle in a recirculating system (ZS560 in Light Enclosure, Aquaneering, San Diego, CA, USA). Culture water pH and conductivity were continually monitored, and pH values ranged between 7.2 and 7.8; electric conductivity between 600 and 800 μS $cm^{-1}$. Adult fish were fed twice a day with *Artemia nauplii* (INVE Aquaculture, Inc., Salt Lake City, UT, USA) and commercial flake

(a combination of Zebrafish Select Diet, Aquaneering, San Diego, CA, USA and Golden Pearls, Artemia International LLC, Fairview, TX, USA). Embryos were obtained by naturally spawning groups of eight to twelve fish in a 1:2 female/male ratio. Spawning time was coordinated using a barrier to separate male and female fish to produce age-matched fertilized eggs. Fertilized eggs were collected within 60 min of spawning.

### 2.3. Bifenthrin exposures and recovery period

Bifenthrin concentrations were chosen based on similar studies that evaluated the impact of bifenthrin on endocrine disruption in fish (Brander et al., 2012; DeGroot and Brander, 2014) and were within the range of concentrations present in aquatic habitats (Weston et al., 2009; Weston and Lydy, 2012; Weston et al., 2014; Weston et al., 2015a; Weston et al., 2015b). Fish from five independent spawns obtained on different days were used to obtain five biological replicates (n=5) for each treatment. Transcriptional and behavioral responses were evaluated across four time points – 1, 3, 5, and 19 dpf – in four experimental groups: three bifenthrin exposures – 1, 10 and 50 ng/L bifenthrin predissolved in methanol (MeOH) – and a vehicle (0.01% MeOH v/v) control group (ASTM, 2014). Exposures were performed from 2 hpf to 5 dpf, and were followed by a 14 day recovery period to 19 dpf. A total of 480 embryos per spawn were split into groups of 30, directly transferred into 16 different glass petri dishes (100×20 mm; 30 embryos/dish; 4 replicates per concentration, one for every investigated time point) containing 60 mL standardized Embryo Medium (Westerfield, 2007), and placed into an incubator at a constant temperature of 28.1 ± 0.7°C, with a 14 h light: 10 h dark photoperiod. Embryos remained in the glass petri dishes throughout the exposure period, and were randomly sampled for transcriptomic and behavioral evaluations. All fish were examined to exclude individual fish with obvious abnormalities. Water changes (80%) were performed daily and physicochemical parameters remained constant throughout the tests: pH 7.6 ± 0.2, dissolved oxygen 8.3 ± 0.3 mg/L and specific conductance of 2195 ± 85 μS $cm^{-1}$.

At 5 dpf, 30 fish from each group were transferred into 1.8 L glass tanks (Mason jars, Erie, PA, USA), containing 1.6 L of culture water from the recirculating system of the adult fish and maintained for a 14 d recovery period until 19 dpf. Fish were fed twice a day with commercial flake from 5 to 19 dpf. Feeding was supplemented with live *Artemia nauplii* starting at 10 dpf, and 80% water changes were performed 60 min after the second feeding took place. To rule out the possibility that differences in transcriptome and behavioral readouts in bifenthrin-exposed vs. vehicle control fish were due to differences in food consumption during the 14 d recovery period, upon completion of the behavioral assays at 19 dpf, all fish were euthanized on ice and transferred into an aluminum dish and placed overnight in a New Brunswick incubator E24 (Eppendorf, Germany) at 70°C. The mean dry weight of fish from each exposure group (n=5) was then measured using a Mettler Toledo AL104 precision scale (Mettler Toledo, Columbus, OH, USA, sensitivity of 0.0001g ± 0.0002g). The data from this experiment is presented in Fig. S5. Physicochemical parameters remained constant throughout the recovery period: pH 7.5 ± 0.1, dissolved oxygen 7.9 ± 0.2 mg/L and specific conductance 875 ± 25 μS $cm^{-1}$.

### 2.4. Transcriptomic assessments

Embryos or larval fish were pooled at each sampling time point (1, 3, 5, 19 dpf) so as to obtain sufficient amounts of RNA for transcriptomic assessments. Specifically, 20 larvae were pooled for samples taken during the acute exposure period and four larvae per replicate were pooled after the recovery period. Prior to sampling, embryos and larvae were euthanized on ice and stored in RNALater (Thermo Fisher Scientific, Waltham, MA, USA) for subsequent RNA extraction.

Total RNA extractions were performed with a Qiagen QiaCube robotic workstation using RNeasy Mini Kit spin columns (Qiagen, Valencia, CA, USA) as per manufacturer's directions. RNA concentrations and extraction efficiency were determined using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Samples were deemed to be of acceptable quality, with 260/280 and 260/230 ratios ranging from 1.98 to 2.24 and 1.78 to 2.20, respectively. Total RNA integrity was additionally visually verified by non-denaturing gel electrophoresis using a 1% (w/v) agarose gel.

Complementary DNA (cDNA) was synthesized using 750 ng total RNA in a reaction with 4 μL Superscript Vilo Mastermix (SuperScript® VILO™ MasterMix, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reactions were incubated for 10 min at 25°C, 60 min at 42°C followed by a 5 min denaturation step at 85°C. Samples were then diluted with nuclease-free water at a 1:5 ratio, to produce the required concentration for quantitative PCR analyses.

We quantified transcript levels of 20 genes, 6 associated with the mTOR signaling pathway and 14 associated with RyR-dependent $Ca^{2+}$ signaling, at three different time points (1, 3 and 5 dpf) during the exposure period and at the end of the recovery period (19 dpf). Transcript levels of target genes were assessed by qPCR using previously designed and validated primers (Frank et al., 2017) (Table 1).

Quantitative PCR was conducted using Power SYBR Green PCR Master Mix (Life-technologies, Carlsbad, CA, USA). Cycling conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C, followed by a thermal ramping stage for dissociation evaluation. Amplification data were analyzed using Sequence Detection Systems software (SDS v2.4.1, Applied Biosystems). GeNorm (Vandesompele et al., 2002) was used to normalize gene expression relative to the reference genes *elongation factor 1 alpha (eef1a1)*, beta-actin2 (*actb2*) and beta2-macroglobulin (β2m), which sustained best stability scores.

### 2.5. Locomotor behavioral assessments

All behavioral assessments were performed using the DanioVision™ high-throughput behavior system (Noldus Information Technology, Inc., Netherlands). Locomotor activity was evaluated at 3 and 5 dpf during the exposure period and post-recovery at 19 dpf. A total of 30 larvae per group (n=5 corresponding to mean data from six larvae from each of five biological replicates) were examined individually at all time points. All behavioral experiments were conducted in the early afternoon to exclude any bias due to possible

intraday-dependent locomotive variation in swimming behavior (MacPhail et al., 2009). Behavioral monitoring of 5 dpf larvae was conducted in a 96-well plate (Falcon™, Corning Inc., Corning, NY, USA), each well contained one larvae and 200 µl of exposure solution. Post recovery assessments were performed in 6-well plates (Falcon™, Corning Inc., Corning, NY, USA) containing 3 ml untreated culture water. Larval fish were transferred from the glass petri dish into polystyrene multi-well plates and allowed to acclimate for 30 min before they were placed into the DanioVision™ observation chamber.

During behavioral assessments, fish were exposed to alternating light/dark stimuli, which is an accepted method for tracking photomotor responses in larval zebrafish (Cario et al., 2011). Fish were initially exposed to light (75% intensity of the system capacity; 1800–2000 Lux) for a 5 min acclimation period, after which behavioral assessments started with an additional 5 min light period (Light 1), followed by a 5 min Dark period (Dark 1), a second 5 min light period (Light 2), a second 5 min dark period (Dark 2) and then finally a 10 minute dark period. Light/Dark preference in zebrafish depends on the stimuli used (Blaser and Peñalosa, 2011). The selected Light/Dark regime was determined after testing different combinations of dark and light periods, durations, and illumination intensities on larvae at 3, 5, and 19 dpf (results not shown). The same behavioral tracking settings were used during locomotive behavioral assessments for fish from the recovery tests, but conducted at reduced illumination (25% intensity of the system capacity; 650–750 Lux) because higher light intensities resulted in permanent burst swimming in 19 dpf fish (data not shown).

### 2.6. Response to predator cue

At 19 dpf, zebrafish were challenged with a suspension of a homogenized tissue from an untreated adult fish, prepared in 10 mL culture water (referred to as a “predator-cue” hereafter) to test behavioral response to a predator cue. This approach ensures contact between the larval fish and the alarm compound, Schreckstoff, released from fish skin after injuries, which is detected by adjacent fish and causes predator avoidance behavior (Jesuthasan and Mathuru, 2008).

Thirty larval fish from each group (6 fish per replicate, n = 5 replicates) were used to assess predatory responses. Fish were placed in 6-well plates (one individual per well) and allowed to acclimate to the plate for 30 min before being transferred into the observation chamber. Once in the Daniovision™ chamber, they had an additional 5 min acclimation before locomotor tracking was initiated. Recording started with a 5-min free swimming evaluation, then 15 µL of the predator cue was added into each well and fish behavior was tracked for another 5 min. Acclimation and response to the predator cue was assessed during a continuous light period (25% intensity of the system capacity; 650–750 Lux).

### 2.7. Statistical Analysis

Differences in gene transcription were tested using one-way ANOVA with significance set at $P < 0.05$, followed by a Tukey Honest Significant Difference (HSD) post-hoc test for pairwise difference. If data did not fit the ANOVA assumptions of normality, a Kruskal-Wallis test was applied ($P < 0.05$), followed by the Dunn’s post-hoc test (p-values are presented in the supplemental material (Table S1). Shapiro-Wilk normality and Bartlett tests

were used to determine which algorithms were appropriate for determining significant differences between treatments.

Data were further analyzed using regression analyses to fit concentration-effect curves. Replicated regression approaches are recommended for evaluating concentration-effect relationships (versus pairwise comparisons) because of their greater statistical power (Isnard et al., 2001; Cottingham et al., 2005). Curve-fitting approaches also provides mechanistic information because they give a prediction of the direction of the responses. The OECD now recommends regression-based estimation procedures for the analysis of toxicity response data. A maximum likelihood estimate 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 the vehicle control (MeOH). 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 $P < 0.05$ (Bolker, 2008). All calculations for the concentration-effect curves were performed using fold-change values. In addition, heat maps of all transcriptomic data were prepared to show functional pathway correlations (supplementary material, Fig. S2).

To assess locomotive behavior, distance moved by each larval zebrafish was recorded during each minute of the 30-min observation period. Using the trapezoidal rule, the area under the curve (AUC) was computed for each fish under 5 lighting conditions: Light 1, 1–5 min; Dark 1, 5–10 min; Light 2, 10–15 min; Dark 2, 15–20 min; and Free swim, 15–30 min (an extended period of Dark 2). Mixed effects regression models, including zebrafish-specific random effects, were used to assess differences between groups defined by three concentrations of bifenthrin and the vehicle control group. Analytical variables were defined to capture differences in area under the curve between Light 1 and Dark 1, Dark 1 and Light 2, and Light 2 and Dark 2 as measures of changes in movement in the transition from light to dark or dark to light. Contrasts for differences between exposed groups and the solvent control group were specified for light and dark conditions separately to compare these transitions and the area under the curve during each of the lighting conditions. A natural logarithmic transformation was best suited to the area under the curve analysis, so as to stabilize the variance and meet the underlying assumptions of the mixed effects model. Due to zeroes, occurring from stagnating fish in the area under the curve, all values were shifted by 0.1 prior to transforming data to a natural logarithmic scale. Concentration (0.1 ng/L, 1 ng/L, 50 ng/L or MeOH), day (3, 5 and 19 dpf), and the transition variables were all compared in the models, including their interactions. Akaike information criterion was used for model selection and Wald tests for comparing groups were used, with a significance level of $p< 0.05$. All analyses were conducted using SAS university edition.

Area under the curve values were used to determine best fitting curves, in order to identify potential concentration-dependent behavioral responses. To allow comparison within endpoints, all datasets were rescaled into a range between 0.0 and 1.0 for graphical illustration using the normalization $x' = \frac{x - xmin}{xmax - xmin}$ at each time point (Figure 1–5). All

untransformed datasets and corresponding graphs are presented as supplementary material (Fig S1).

## 3. Results

### 3.1. Molecular responses to bifenthrin

Exposure to low (ng/L) concentrations of bifenthrin resulted in time-dependent increases in transcription of both mTOR and RyR signaling pathway-associated genes during early development (at 1, 3, and 5dpf); however, there was an inverse relationship between bifenthrin and transcriptomic changes at 19 dpf following a 14 d recovery period (Figure 1–3). At 1 dpf, developmental exposure to bifenthrin caused a significant concentration-dependent increase in transcripts of *ryr1b*, the RyR paralog predominantly found in fast twitch muscles (Figure 1). Transcriptional responses of genes involved in the mTOR pathway were similarly increased. Data demonstrated either linear or quadratic (non-monotonic) responses, with increased transcription at 1 dpf of *mtor,* mTOR complex 2 (mTORc2) member *rictora,* or the upstream member *rheb* in fish exposed to bifenthrin at 50 ng/L. (Figure 2).

At 3 dpf, there was also a correlation between increased transcription of *ryr2a* and *ryr3,* the RyR-paralogs predominantly transcribed in the nervous system, and higher bifenthrin concentrations (Figure 1). Furthermore, strong concentration-dependent increases were observed in all calcium channel genes, with transcription of *cacna1c* and *cacna1sb* responding significantly. *Cacna1c* also showed significant upregulation in the 50ng/L exposure group in the ANOVA calculation, compared to all other treatments (Figure 3, Table S1). Another impact on calcium signaling systems was detected in the form of a significant quadratic response of the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) *atp2a2a,* a paralog predominantly expressed in slow twitch muscle and heart tissue. This quadratic response shows decreased transcription of *atp2a2a* in larval fish from the lowest bifenthrin concentration and increased transcription in the 50 ng/L treatment, relative to vehicle control fish (Figure 3). Similar to observations at 1 dpf, *mtor* transcripts were significantly correlated with bifenthrin concentration at 3 dpf.

Larvae sampled on the last day of exposure (5 dpf) provided further evidence that bifenthrin altered transcription of signaling molecules in the calcium signaling network, with significant concentration-dependent increases in transcription of *atp2a1* and *cacna1c,* a calcium transporter and ion channel, respectively, and quadratic responses in *ryr2b* and *cacna1sa* (Figure 1 and 3). There were no significant changes in the transcription levels of RyR-paralogs at 5 dpf (Figure 1). As observed at 1 and 3 dpf, at 5 dpf there were significant transcriptional differences in *mtor*, as well as the downstream signaling member *eif4ebp1*, both of which correlated with bifenthrin concentration in a concentration-dependent manner (Figure 2).

Opposite to transcriptional changes observed during the exposure period, the transcription of most RyR paralogs decreased in a concentration-dependent manner in 19 dpf larvae. Transcripts of *ryr2b* were the only RyR genes that remained elevated during both exposure and recovery periods in fish exposed to bifenthrin at 50 ng/L (Figure 1). In contrast to

measurements during the exposure period, there were no significant differences in transcription of genes coding for SERCA pumps and voltage-gated $Ca^{2+}$ channels post recovery (Figure 3). There were concentration-dependent decreases in transcription at 19 dpf in all mTOR signaling pathway genes investigated in this study (Figure 2), with significant differences determined for *mtor*, as well as for the mTORc1 partner, *rptor,* and the downstream target, *eif4ebp1*.

### 3.2. Locomotor assessments

Freshly hatched larval fish (< 5 dpf) in all groups moved more during dark periods compared to light periods (illustrated for vehicle control fish, Figure 4A). This canonical photomotor behavior changed in all groups with ongoing development, such that by 19 dpf, fish swam greater distances during light periods (illustrated for vehicle control fish, Figure 4B).

Swimming performance was affected by bifenthrin exposure, but this was only significant in 19 dpf fish following the 14 d post-recovery period (Figure 4C, D). The majority of newly hatched larvae at 3 dpf showed little movement during light periods. Dark phases, however, triggered comparable swimming responses in all exposure groups, with occasional swimming bursts. At 5 dpf, larvae showed a tendency towards less movement with exposure to higher bifenthrin concentrations during both light phases and increased swim behavior in the Dark 1, Dark 2 and final Free swim period (Figure 4D). However, no statistically significant differences were detected between groups during the acute exposure period at 3 or 5 dpf. In contrast to the lack of bifenthrin effects on the photomotor response during acute exposure, there were significant bifenthrin-related differences in swimming performance in post-recovery larvae at 19 dpf (Figure 4D). Specifically, during a second light period challenge (Light 2), fish exposed to bifenthrin at 1 or 10 ng/L were significantly more mobile relative to either vehicle controls or the 50 ng/L bifenthrin groups. Similar patterns were observed in Dark 2 and the Free swim period, whereas no significant bifenthrin effect was observed during Light 1 and Dark 1.

The mixed model approach used to highlight differences between the single treatments integrates distance moved along with the transition between light and dark stimuli, providing a robust behavioral analysis. Calculations in the mixed model approach demonstrated increased movement for the larval fish from the low bifenthrin treatments (1 and 10 ng/L) during Dark 2 and Free swim on 19 dpf, showing significant differences when compared to the vehicle control (Figure 4). Furthermore, a significant decrease in movement was observed in fish from the 50 ng/L bifenthrin group relative to the solvent control group during Light 1 on 19 dpf.

### 3.3. Response to a predator cue

The response to a predator cue was assessed at 19 dpf after a 14 d recovery period. There were no significant differences in swimming behavior during the initial 5 min acclimation period, but as noted in the light/dark locomotor tests, there was a tendency for increased movement in larvae exposed to 1 and 10 ng/L bifenthrin (Figure 5). Larvae from the control group responded with immediate immobilization (freezing) when challenged with a predator cue. Over the following 5 min they returned to normal (baseline) swimming activity. The

majority of bifenthrin-exposed larvae also responded with initial freezing, but returned to baseline swimming behavior more rapidly than vehicle control individuals. Concentration-effect fitting curves demonstrated a significant non-monotonic, quadratic concentration-effect response, with increased movement in larvae exposed to 1 and 10 ng/L bifenthrin (Figure 5).

The increased movement of larval fish from the 1 ng/L treatment was further confirmed by the mixed model algorithm, which indicated significantly increased activity when compared to controls. While the 10 ng/L bifenthrin group showed increased activity as well, the data were not significantly different when assessed using the mixed model algorithm.

## 4. Discussion

While prior studies have investigated micromolar bifenthrin concentrations exposure impacts on zebrafish development (Jin et al., 2009), to the best of our knowledge, this is the first study describing impacts of bifenthrin on neurodevelopmental processes in zebrafish exposed to low (ng/L) concentrations, which correspond to pM concentrations. The combined assessment of molecular and behavioral endpoints during chemical exposure from 2 to 5 dpf and after a recovery period reveals distinct responses at different levels of biological organization, and illustrates delayed changes in behavior. Specifically, we observed that in the developing zebrafish, bifenthrin exposure during early development acutely upregulated transcription of mTOR and RyR-dependent signaling molecules, and caused delayed downregulation of these transcripts and delayed behavioral deficits. The behavioral assessments conducted in this study, which included challenging fish with visual and olfactory stimuli, have been demonstrated to be strong indicators of neuronal dysfunction (Tierney, 2011). Perturbations of mTOR- and RyR-dependent $Ca^{2+}$ signaling in the developing brain have previously been linked to adverse neurodevelopmental outcomes in rodent models (Mori et al., 2000; Bers, 2004; Kumar et al., 2005; Berridge, 2006; Lee et al., 2011; Bowling et al., 2014; Tang et al., 2014). Collectively, these data suggest a potential link between bifenthrin effects on transcription of mTOR and RyR-dependent signaling molecules and bifenthrin effects on behavior.

### 4.1. Specific gene transcription effects

Transcripts of RyR signaling molecules showed overall concentration-dependent increases during the exposure period, and subsequent decreases after the recovery period. A significant concentration-dependent response was observed at 3 dpf for *ryr2a*, a RyR-paralog found predominantly in nervous tissue during zebrafish development (Wu et al., 2011). A similar response was observed for *ryr3*, also known to be expressed in zebrafish nervous tissues (Darbandi and Franck, 2009). This is of particular interest since the developmental stage at which increased transcription of *ryr2a* and *ryr3* were observed corresponds to the timing of complex neuronal circuit formation and significant synaptogenesis in the developing zebrafish brain (Saint-Amant and Drapeau, 1998; Brustein et al., 2003). Earlier studies demonstrated that chemical-induced increases in RyR-dependent $Ca^{2+}$ signaling during development were associated with enhanced dendritic outgrowth, increased synaptic density and impaired cognitive behavior (Yang et al., 2009; Wayman et al., 2012b; Wayman

et al., 2012a; Lesiak et al., 2014). Additional concentration-dependent impacts on $Ca^{2+}$ homeostasis at 3 dpf included the upregulation of the SERCA pump *atp2a2a*, as well as voltage gated calcium channels *cacna1c* and *cansa1sb*, illustrating pervasive effects on calcium homeostasis (Figure 3). It is not clear how our findings relate to previous reports of decreased plasma calcium levels in freshwater catfish (*Heteropneustes fossilis*; 37–42g) following a 96 h exposure to 5.76 μg/L and 28 d exposure to 1.44 μg/L of the pyrethroid cypermethrin (Mishra et al., 2005). However, our findings are consistent with a previous study demonstrating that bifenthrin acutely increases calcium oscillations in cultured cortical rat neurons (Cao et al., 2014). Interestingly, bifenthrin effects in primary rat cultures were observed at nanomolar concentrations, whereas we observed bifenthrin effects on transcription of RyR-relevant genes in zebrafish at picomolar concentrations. This discrepancy in effective concentration levels is in contrast to the commonly held perception that *in vitro* model systems are more sensitive than *in vivo* model systems. While the biological explanation for this difference is unknown, it perhaps reflects the observation that fish are more vulnerable than mammals to pyrethroid toxicity (Glickman and Lech, 1982). Collectively, these observations support a generalized effect of pyrethroids on $Ca^{2+}$ homeostasis and signaling *in vivo* as well as *in vitro*.

Concentration-dependent changes were observed in five of the six genes investigated within the mTOR pathway (Figure 2). The most extensive impacts where observed in the gene coding for mTOR, with significant concentration-dependent responses observed at all time points. At 1 dpf, the mTORC2 components, *mtor and rictora*, as well as the upstream signaling molecule, *rheb,* were upregulated in a concentration-dependent manner, indicating activation of the entire signaling cascade (Sarbassov et al., 2005b; Guertin et al., 2006; Bai et al., 2007). With ongoing exposure, a concentration-dependent significant increase in the downstream target, *eif4ebp1*, which encodes for a protein that inhibits the translational mechanisms of mTOR signaling (Ma and Blenis, 2009) was observed at 5 dpf. Upregulation of *eif4ebp1* has the potential to diminish pathway activation, since transcription of mTOR upstream members with activating function were consistently upregulated during earlier time points. Concentration-dependent decreases in transcription were observed for all mTOR pathway members after the recovery period, with the most robust changes observed for the mTORC2 members, *mtor* and *rictora*, and *eif4ebp1*. The most consistently decreased transcripts correlated with the higher bifenthrin concentrations.

### 4.2. Transcription vs. behavior

Here, we showed a biphasic transcriptomic response of genes involved in mTOR and RyR-dependent $Ca^{2+}$ signaling pathways: transcription of these genes was generally increased during exposure with the most robust increases in transcription was detected in the fish exposed to the highest concentration of bifenthrin tested (50 ng/L). However, there was a clear trend of decreased transcription after a 14 d recovery from bifenthrin. Similar inverse relationships in transcriptomic responses have been observed after a recovery period in fathead minnow larvae, following short term (24 h) exposure to bifenthrin (Beggel et al., 2011). Given the extensive use of bifenthrin and its known neurotoxic effects, surprisingly few other studies have examined the effects of low (ng/L) concentrations in vertebrates. These include studies of the inland silverside (*Menidia beryllina*) (Brander et al., 2012;

DeGroot and Brander, 2014; Brander et al., 2016b), one of which described the integrated effects of a 14 d exposure to low levels of bifenthrin on transcription of genes representative of endocrine function and on reproduction (Brander et al., 2016b). Higher concentrations of bifenthrin have been shown to interfere with nervous system function. Thus, at 1.5 μg/L, bifenthrin interferes with dopaminergic signaling in juvenile rainbow trout (Crago and Schlenk, 2015), and at concentrations ranging from 75 ng/L to 4 μg/L, a 24 h exposure to bifenthrin decreased transcription of genes related to metabolism, growth, stress response, muscular and neuronal activity in larval fathead minnows (*Pimephales promelas*)(Beggel et al., 2011), suggesting broader impacts of bifenthrin exposure on organismal development at higher concentrations than those we tested here.

Bifenthrin did not cause significant behavioral changes at 5 dpf in our study. However, post-recovery behavioral assessment data demonstrate significant differences in both photomotor behavior and locomotor response to a predator cue at 19 dpf in zebrafish exposed to lower bifenthrin concentrations. It has previously been reported that the pyrethroid deltamethrin increased incidence of eye cataracts in adult Nile tilapia, *Oreochromis niloticus* (El-Sayed and Saad, 2008), suggesting the possibility that bifenthrin-induced behavioral deficits in response to light-dark stimuli are secondary to ocular toxicity. However, we think this is unlikely to be the case, because: (1) we did not observe any eye cataracts in any of the zebrafish in our studies, and (2) eye cataracts were observed in tilapia exposed to deltamethrin at 1.46 μg/L for 28 consecutive days, whereas we observed significant hyperreactivity in 19 dpf zebrafish exposed to bifenthrin at low (1 and 10 ng/L) but not high (50 ng/L) pM concentrations of bifenthrin for only 5 consecutive days with 14 days of “washout”. Moreover, the behavioral phenotype we observed in zebrafish exposed to bifenthrin is consistent with previous reports demonstrating hyperactivity associated with pyrethroid exposure in fish and rats (Jin et al., 2009; Richardson et al., 2015).

Another question is whether the delayed behavioral effects of bifenthrin reflect effects of bifenthrin on neurodevelopmental processes, or whether bifenthrin is still present in the brain at 19 dpf. In zebrafish larvae exposed to bifenthrin at 2 or 20 μg/L, the half-life of bifenthrin was found to be 15.9 or 38.5 hours, respectively (Tu et al., 2014). Assuming that bifenthrin is completely eliminated within 5 half-lives, it seems likely that behavioral deficits observed at 19 dpf, which is 14 d after exposure ended, are attributable to disruption of neurodevelopment. Interestingly, recent reports in a rodent model indicates that in the adult organism, bifenthrin elicits behavioral deficits during the exposure period, but not after a recovery period (Syed et al., 2018), suggesting differential effects of bifenthrin on the developing *versus* the mature brain.

When comparing results from molecular and behavioral assessments, the strongest transcriptional changes were correlated with higher chemical concentrations, whereas the greater impacts on behavior were observed in fish exposed to bifenthrin at 1 and 10 ng/L. These observations suggest several possibilities: (1) transcriptional changes in calcium-dependent signaling pathways are not causally related to behavior changes; (2) subtle differences observed at the molecular level contribute to, or manifest as, delayed effects as determined via behavioral assessments whereas robust increases in transcripts from mTOR and RyR-dependent $Ca^{2+}$ signaling genes in the group exposed to 50 ng/L bifenthrin may be

compensatory responses. The non-monotonic concentration-effect relationship for bifenthrin effects on behavioral endpoints was confirmed by curve fitting (which identified quadratic responses as the best fit) and by a mixed model algorithm. The biological reason(s) for the non-monotonic concentration-related effects of bifenthrin on behavior are not known. Non-monotonic concentration-effect relationships have been observed for bifenthrin effects on other endpoints. For example, low (ng/L) concentrations of bifenthrin have caused non-monotonic responses in gene transcription of endocrine-related genes in *inland silversides* (Brander et al., 2016b), with strongest effects observed with the lowest applied concentration of 0.5 ng/L. Similar non-monotonic concentration-effect relationships have been reported for PCB 95, a flame retardant that interferes with RyR dependent $Ca^{2+}$ signaling to enhance dendritic growth and interfere with learning and memory at lower but not higher concentrations (Yang et al., 2009; Wayman et al., 2012b; Wayman et al., 2012a).

Further research to assess protein levels of altered transcripts, as well as neurodevelopmental processes influenced by RyR- and mTOR-dependent signaling, such as axonal outgrowth, dendritic arborization and synapse stabilization in relevant brain regions, could help to identify the mechanistic link(s) between developmental exposure of bifenthrin, altered transcription of RyR- and mTOR signaling molecules, and delayed behavioral alterations.

### 4.3. Low (ng/L) concentrations and concentration-effect relationships

This study provides evidence for concentration-dependent neurobehavioral responses elicited by developmental exposures to low (ng/L) concentrations of bifenthrin. Environmental concentrations of chemical pollutants are generally below acutely toxic levels; however, potential sublethal toxic effects are of increasing concern (Sandahl et al., 2005; Geist et al., 2007; Connon et al., 2012). Persistent impacts on behavior following a developmental exposure to bifenthrin at 1 and 10 ng/L were identified in the present study. These findings illustrate the importance of including low concentrations in experiments that seek to evaluate the effects of environmentally relevant levels of persistent chemicals, and the need to focus on delayed effects. Given the small number of studies incorporating low (ng/L) concentrations of pyrethroid insecticides, but the strong effects measured following exposure of developing fish to these low levels, there are likely a larger number of sublethal effects resulting from exposures at these low concentrations that may adversely impact the overall health status of an organism that have yet to be determined.

## 5. Conclusion

In this study, RyR and mTOR signaling pathways were observed to be impacted by developmental exposures to bifenthrin in a concentration-dependent manner, although the direction of change varied depending on when it was measured. During the developmental exposure period, transcription was generally upregulated in the bifenthrin exposure groups, whereas following a recovery period, transcription was largely decreased, particularly in fish exposed developmentally to the higher bifenthrin concentrations. Collectively, these data confirm that low (ng/L) concentrations of bifenthrin alter transcription of key genes involved in neurodevelopment. Behavioral assessments provided evidence that developmental exposure to bifenthrin also altered neuronal function, evidenced as hyperactivity in 19 dpf

fish exposed to the lower bifenthrin concentrations (1 ng/L and 10 ng/L). Our findings demonstrate the importance of conducting toxicological studies at low (ng/L) concentrations levels of environmental relevance. We further provided evidence that developmental exposures that do not cause detectable effects on behavior during acute exposure can cause delayed behavioral deficits in the older organism. Because neurodevelopment is highly conserved across species, and given the wide-spread exposure of humans, including children, to environmental levels of bifenthrin, these studies also identify bifenthrin as a potential environmental risk factor for NDDs, and in particular those characterized by hyperreactivity, such as attention deficit hyperactivity disorder.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

**Funding**

This project was supported by the National Institute of Environmental Health Sciences (R01 ES014901 to PJL and F32 ES024070 to GWM); the United States Environmental Protection Agency (RD 835550 to PJL); and the Bayerische Forschungsstiftung (contract no. DOK-169-14 to J. Geist, which provided a fellowship to DFF). The sponsors were not involved in the study design, the collection, analysis, and interpretation of data, in the writing of the report or in the decision to submit the paper for publication.

## Abbreviations

| | |
|---|---|
| **dpf** | days post-fertilization |
| **mTOR** | mechanistic target of rapamycin |
| **NDD** | neurodevelopmental disorder |
| **PCB** | polychlorinated biphenyl; |
| **RyR** | ryanodine receptor |

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## Highlights

- Environmentally relevant levels of bifenthrin alter zebrafish development
- Bifenthrin alters transcription of ryanodine receptor and mTOR signaling molecules
- Bifenthrin causes delayed hyperactivity in zebrafish

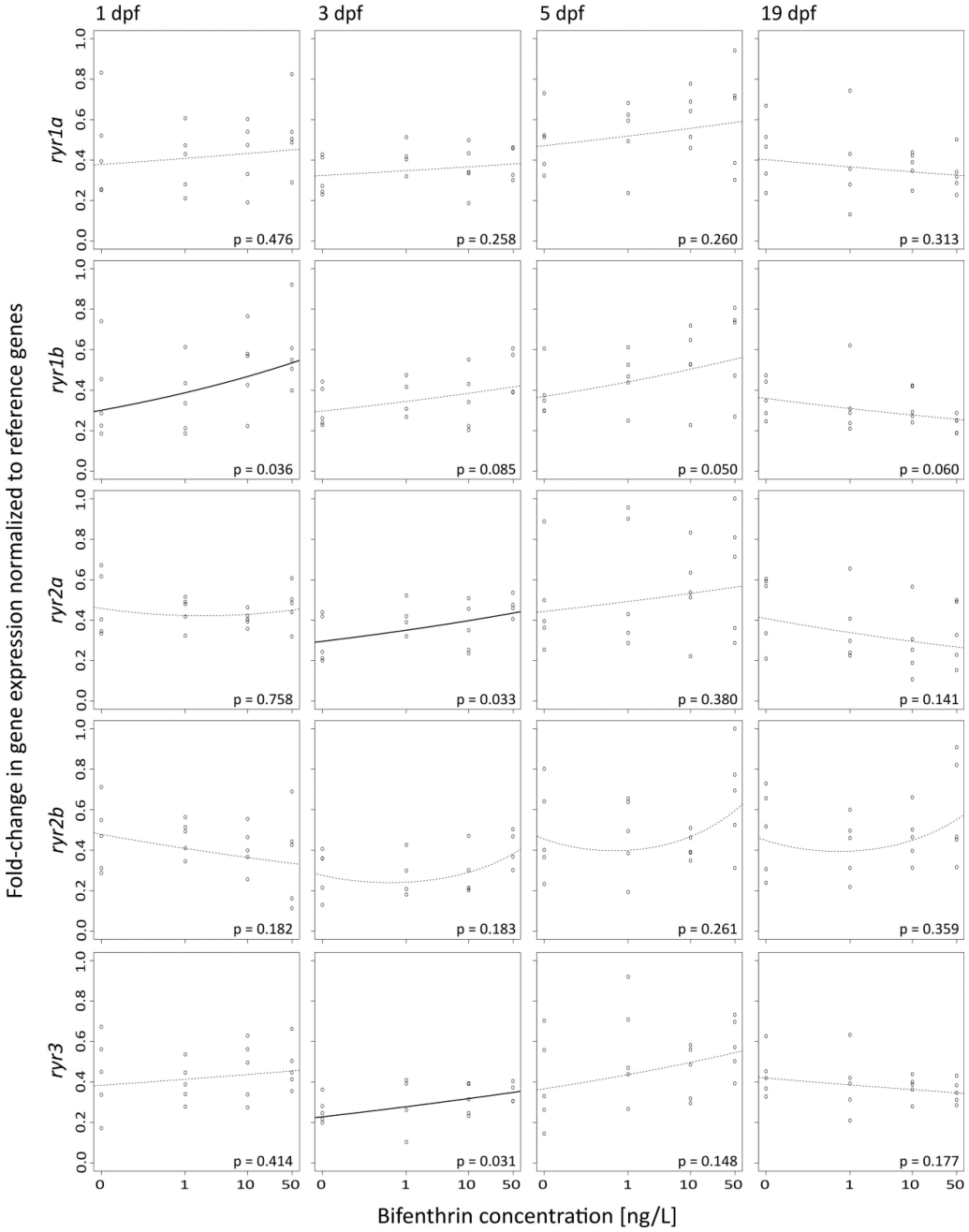


**Fig. 1. Transcriptional changes ryanodine receptor (RyR) paralogs in zebrafish larvae exposed to varying concentrations of bifenthrin from 1 to 5 dpf**

Each dot represents the fold change value of a single biological replicate (n=5 biological replicates), normalized to the average of the reference genes *actb2, b2m* and *eef1a1* within the same sample. Data are presented on a log10 X + 0.05 axis. For data in each panel, five curves (linear, unimodal 1, unimodal 2, sigmoidal and quadratic) were assessed for best fit using the 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 fitting curves are shown in

the panel. Fold-change values were rescaled between 0 and 1 with a normalization calculation for each time point, to facilitate comparison between genes. Graphs illustrating the actual values (Figure S1) and heat maps of the data (Figure S2) are provided in the supplementary material.

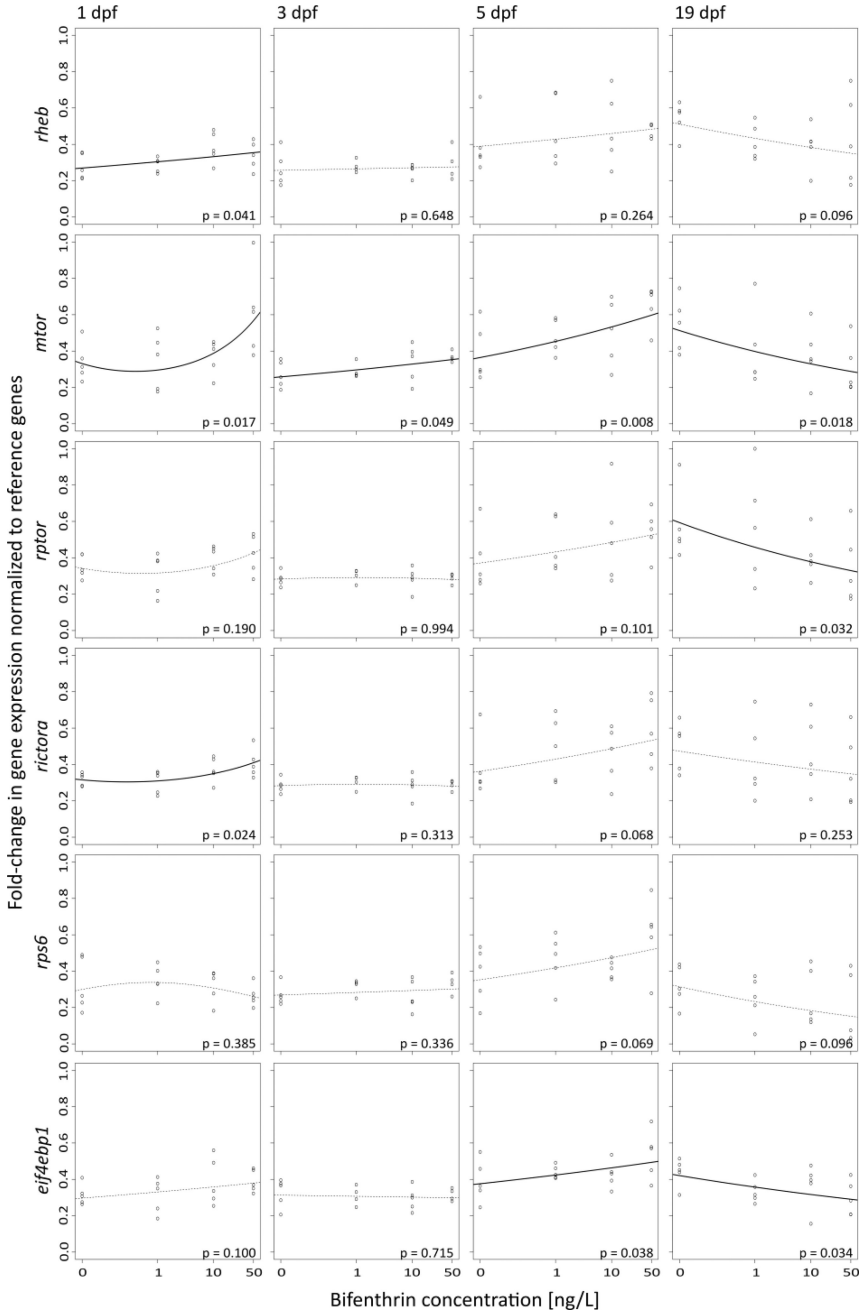


**Fig. 2. Transcriptional changes for mTOR signaling molecules in zebrafish larvae exposed to varying concentrations of bifenthrin from 1 to 5 dpf**

Each dot represents the fold change value of a single biological replicate (n=5 biological replicates), normalized to the average of the reference genes *actb2, b2m* and *eef1a1* within the same sample. Data are presented on a log10 X + 0.05 axis. For data in each panel, five curves (linear, unimodal 1, unimodal 2, sigmoidal and quadratic) were assessed for best fit using the 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 fitting curves are shown in

the panel. Fold-change values were rescaled between 0 and 1 with a normalization calculation for each time point, to facilitate comparison between genes. Graphs illustrating the actual values (Figure S1) and heat maps of the data (Figure S2) are provided in the supplementary material.

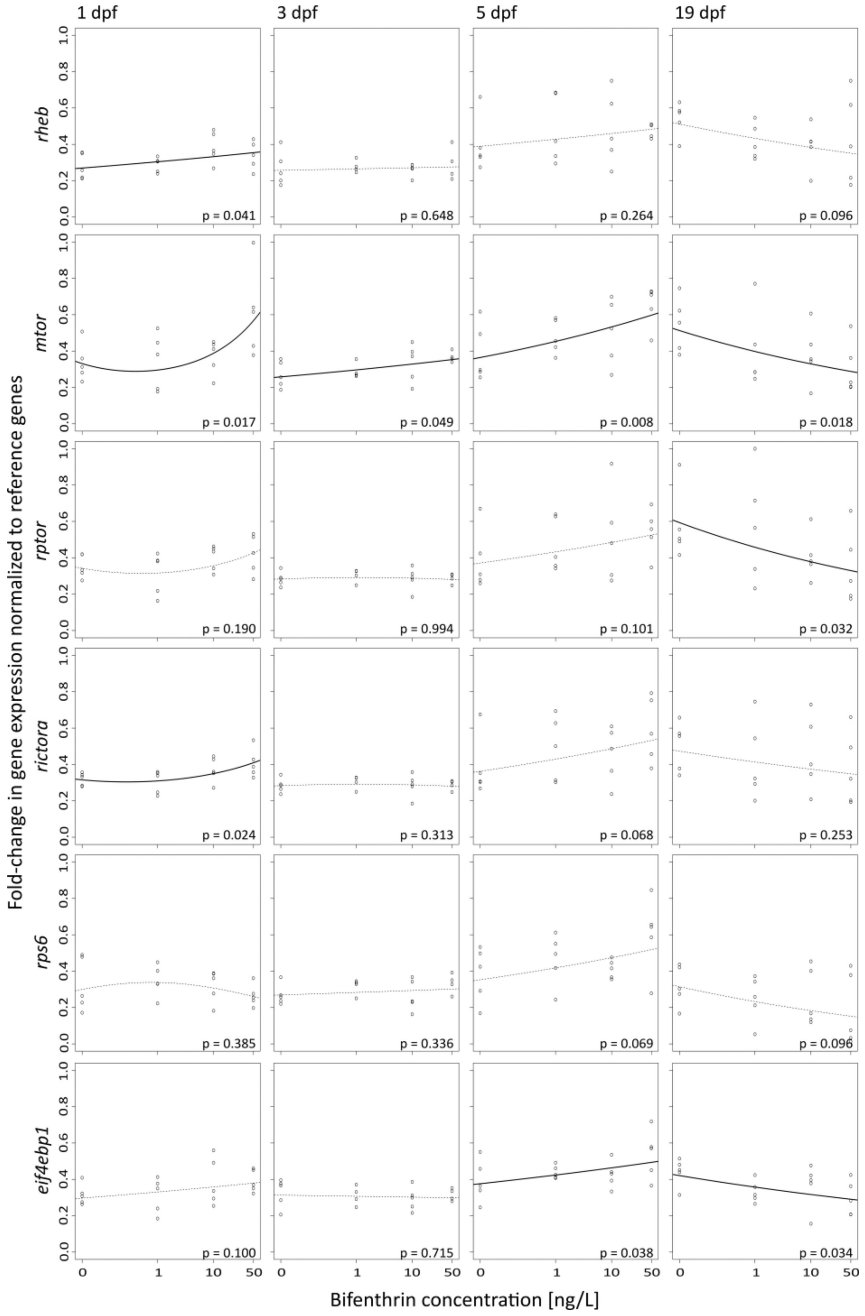


**Fig. 3. Transcriptional changes for SERCA pumps and voltage-gated Ca2+ channels in zebrafish larvae exposed to varying concentrations of bifenthrin from 1 to 5 dpf**

Each dot represents the fold change value of a single biological replicate (n=5 biological replicates), normalized to the average of the reference genes *actb2, b2m* and *eef1a1* within the same sample. Data are presented on a log10 X + 0.05 axis. For data in each panel, five curves (linear, unimodal 1, unimodal 2, sigmoidal and quadratic) were assessed for best fit using the 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 fitting curves are shown in

the panel. Fold-change values were rescaled between 0 and 1 with a normalization calculation for each time point, to facilitate comparison between genes. Graphs illustrating the actual values (Figure S1) and heat maps of the data (Figure S2) are provided in the supplementary material. * Significantly different from control, as identified using one-way ANOVA ($p < 0.05$).

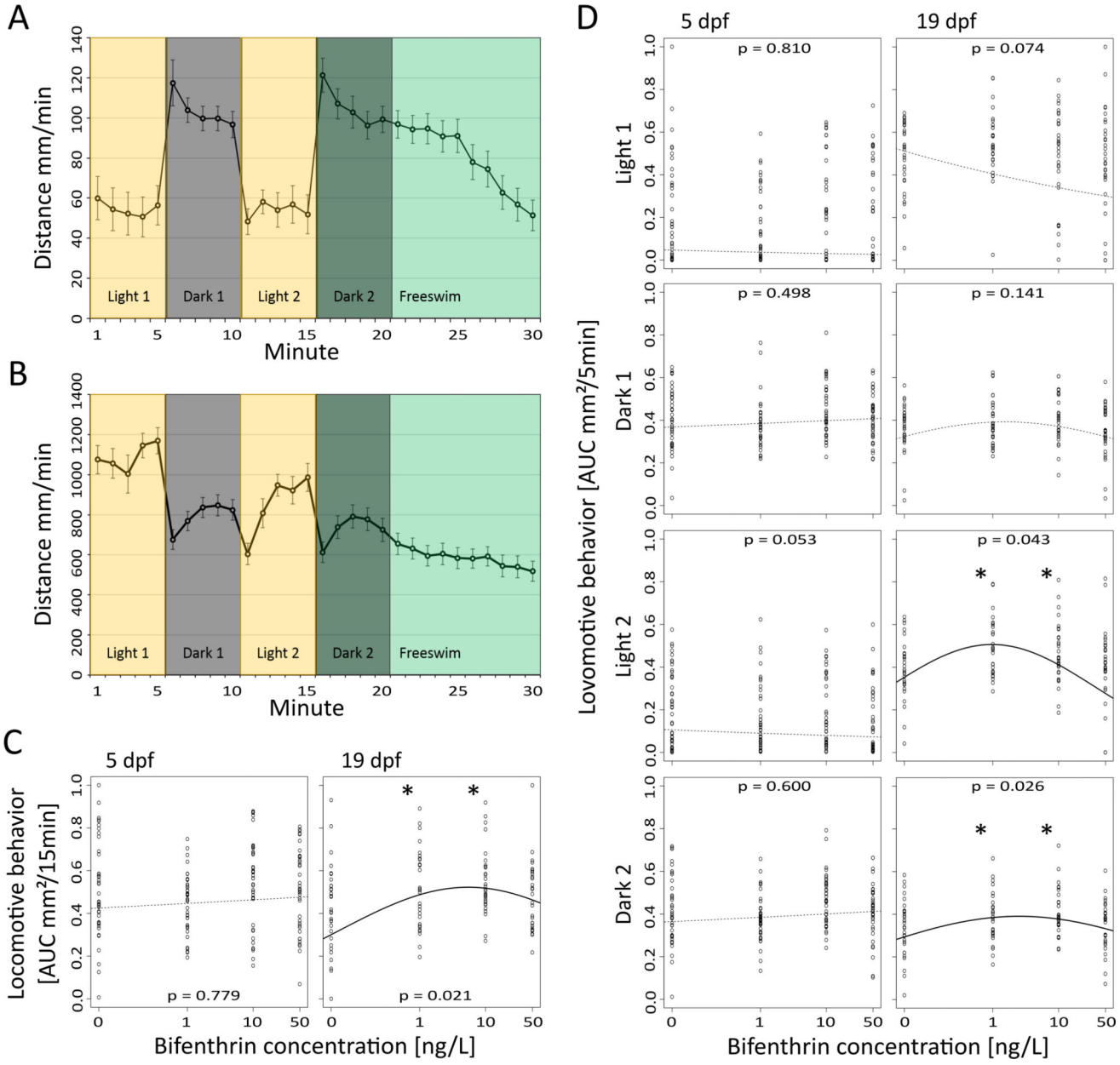


**Fig. 4. Developmental exposure to bifenthrin altered responses of zebrafish in the light-dark locomotor behavioral assay at 19 but not 5 dpf**

Locomotor behavior of zebrafish in alternating periods of light and dark was assessed in zebrafish exposed to varying concentrations of bifenthrin from 1–5 dpf. Locomotor behavior in vehicle control fish during alternating light and dark periods at **(A)** 5 dpf and at **(B)** 19 dpf after a 14-day recovery period is shown as the mean distance in mm moved per minute ± SEM (n = 30 individual larval fish). **(C, D)** Locomotive behavior of 5 dpf (left) and 19 dpf (right) air larval zebrafish exposed to vehicle (0 ng/ml bifenthrin) or varying concentrations of bifenthrin with the distance moved presented as the area under the curve (AUC). Data are presented on a log10 X+ 0.05 axis, and each dot represents one larval fish (n=3o per treatment). **(C)** Data collected during an extended Dark 2 period (15 min “Free swim”). **(D)**

Data collected during alternating light and dark periods, each lasting 5 min. AUC values were rescaled between 0 and 1 with a normalization calculation for each day (determined separately for the Free swim period) to facilitate comparison between light and dark periods at any given time point (graphs with raw values are presented in the supplementary material). Five concentration-effect curves (linear, unimodal 1, unimodal 2, sigmoidal, and quadratic) were fit using a maximum likelihood approach. Curves shown as a solid line are significantly better fits than a null intercept-only model ($p < 0.05$); curves represented by a dashed line are the best-fit of the five curve options (lowest p-value), but not significantly better than the null model (all p-values for the fitted curves are also shown in the graphs representing one period). *Significantly different from control as identified using a mixed model algorithm ($p< 0.05$).

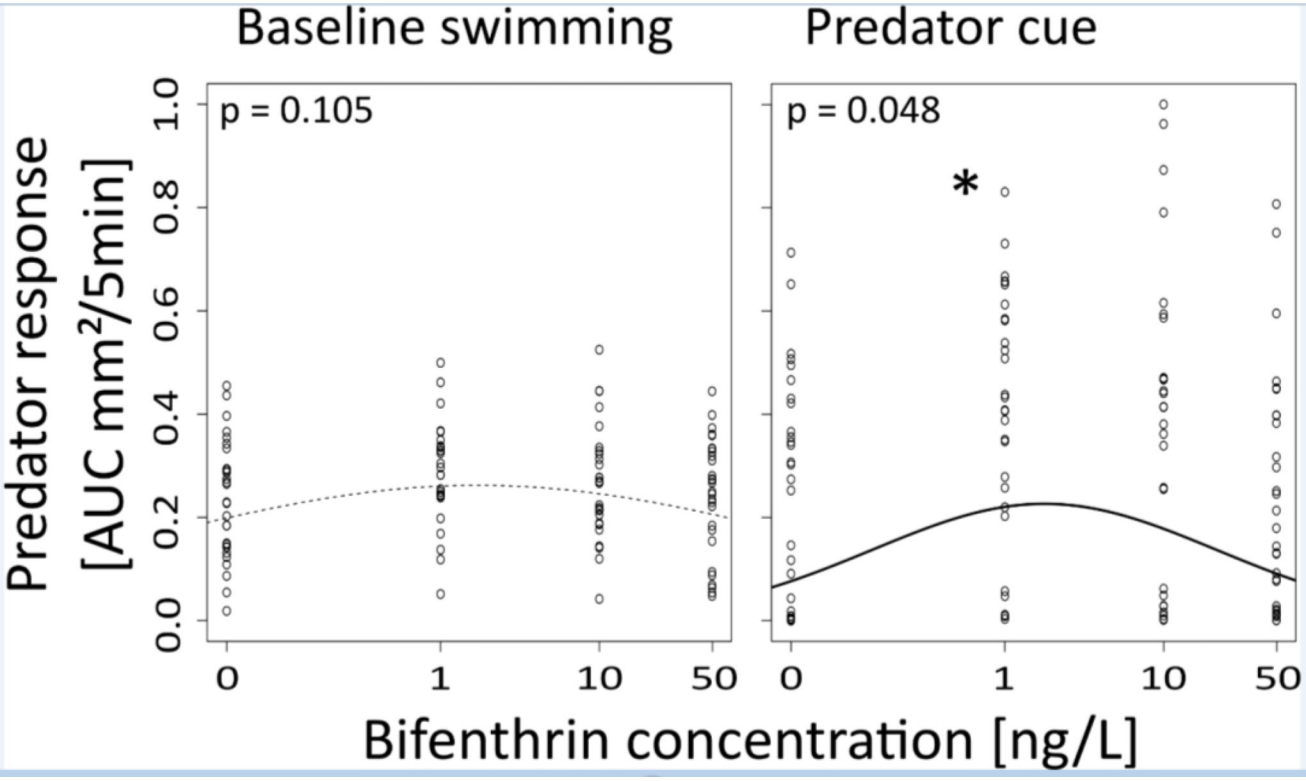


**Fig. 5. Response to an olfactory predator cue in 19 dpf zebrafish exposed to bifenthrin from 1 to 5 dpf**

Swimming was tracked for 5 min during an acclimation period (baseline swimming), and after challenge with a predator cue. Each treatment (presented on a log10 X+ 0.05 axis) included n=30 larval fish, each represented by a single dot. The distance moved is presented as the area under the curve (AUC) during a time interval of 5 min. AUC values were rescaled between 0 and 1 using a normalization calculation (graphs with raw values are presented in the supplementary material). Five concentration-effect curves were fit using a maximum likelihood approach (linear, unimodal 1, unimodal 2, sigmoidal, and quadratic). Curves shown as a solid line are significantly better fits than a null intercept-only model ($p < 0.05$); curves represented by a dashed line are the best-fit of the five curve options (lowest p-value), but not significantly better than the null model. *Significantly different from control, as identified using a mixed model algorithm ($p< 0.05$).

## Table 1

Gene-specific primers for RyR- dependent $Ca^{2+}$ signaling and the mTOR pathway.

| Gene name | Gene code | Primer (5'->3') | Accession # | Amplification Efficiency % |
|---|---|---|---|---|
| **Reference genes** | | | | |
| Beta-actin2 | *actb2* | **F:** AAGCAGGAGTACGATGAGTC<br>**R:** TGGAGTCCTCAGATGCATTG | NM_181601 | 101.7 |
| Beta-2-Microglobulin | *b2m* | **F:** CCACTCCGAAAGTTCATGTGT<br>**R:** ATCTCCTTTCTCTGGGGTGAA | NM_131163.2 | 93.7 |
| Elongation factor 1 alpha 1 | *eef1a1* | **F:** GATGCACCACGAGTCTCTGA<br>**R:** TGATGACCTGAGCGTTGAAG | AY422992 | 99.1 |
| **Target genes** | | | | |
| Ryanodine receptor 1a | *ryr1a* | **F:** TCCTGCTACCGAATCATGTG<br>**R:** GCCTCTCTGCATGTGGAGTT | XM_009305502 | 97.0 |
| Ryanodine receptor 1b | *ryr1b* | **F:** AAGAAATCGGGCATCTGGCA<br>**R:** TGAGTTGTTCGGAGGTGACG | NM_001102571 | 90.6 |
| Ryanodine receptor 2a | *ryr2a* | **F:** AGGACTCAAGCCAAATCGAG<br>**R:** TCACGACCATGTCCTTCTGA | XM_009300703 | 107.0 |
| Ryanodine receptor 2b | *ryr2b* | **F:** TCCTTTAGTCATTTTTAAGCGAGA<br>**R:** GTCCATCGAACTCCAGTTTACG | XM_017351708 | 97.9 |
| Ryanodine receptor 3 | *ryr3* | **F:** TCTTCGCTGCTCATTTGTTG<br>**R:** AGACAGGATGGTCCTCAAGGT | AB355791 | 103.9 |
| ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 | *atp2a1* | **F:** TGAAGCTTTCCTTCCCAGTCA<br>**R:** TCTGCGTGGAGTGCTGTTTTA | BC085636 | 95.1 |
| ATPase, Ca++ transporting, cardiac muscle, slow twitch 2a | *atp2a2a* | **F:** TCTTGACTTTAGTGCATGTGTTGT<br>**R:** TCGCTCCTAATGTTTGAGCTTCT | BC045327 | 90.4 |
| calcium channel, voltage-dependent, L type, alpha 1C subunit | *cacna1c* | **F:** ATATCTTCAGGCGGTCTGGTG<br>**R:** GACTGTGGGAAGGAGACGTG | NM_131900 | 100.3 |
| calcium channel, voltage-dependent, L type, alpha 1S subunit, a | *cacna1sa* | **F:** GATAGCAGAGCGGACAGGAC<br>**R:** TGCATGCTGGGAAATGTGGT | NM_001146150 | 98.2 |
| calcium channel, voltage-dependent, L type, alpha 1S subunit, b | *cacna1sb* | **F:** GCATTCGGTCCGAGAAGCTA<br>**R:** TGACCCGACCAGGATGATCT | NM_214726 | 93.4 |
| Ras homolog enriched in brain | *rheb* | **F:** TTGGACATGGTGGGGAAAGT<br>**R:** TTCACAGCTGATCACTCGCT | NM_200729 | 95.9 |
| Mechanistic target of Rapamycin | *mtor* | **F:** ATGGTCACTGGCCTGAAGTG | NM_001077211 | 102.1 |

| Gene name | Gene code | Primer (5'->3') | Accession # | Amplification Efficiency % |
|---|---|---|---|---|
| | | **R:** GTGCACGTGGCGTATCAATC | | |
| Raptor regulatory associated protein of MTOR | *rptor* | **F:** TTCATCAAGCTGGCGGATCTC<br>**R:** CATCTTCCTGGTGCGTGGA | XM_005157354 | 96.5 |
| RPTOR independent companion of MTOR, complex 2a | *rictora* | **F:** ATCTGATCCGTGACAGCAGC<br>**R:** CCAATCGGAGGGCTTGAGTT | XM_009301197 | 102.2 |
| Ribosomal protein S6 kinase 1 | *rps6* | **F:** TCTGAGCCCTTACGCTTTCG<br>**R:** TCTGTTGGGTGGGGAGATGAT | EF373681 | 100.9 |
| eukaryotic translation initiation factor 4E binding protein 1 | *eif4ebp1* | **F:** ACATGGGGGACGTTTTCACA<br>**R:** GGAGTTGGATTTCCCCCACA | NM_199645 | 102.4 |